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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

0061/00091

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)

09/622257

INTERNATIONAL APPLICATION NO.

INTERNATIONAL FILING DATE

PRIORITY DATE CLAIMED

PCT/FR99/00316

12 February 1999

13 February 1998

#### TITLE OF INVENTION

NUCLEIC ACID COMPRISING THE SEQUENCE OF A STRESS-INDUCIBLE PROMOTER AND A SEQUENCE OF A GENE ENCODING A STILBENE SYNTHASE

#### APPLICANT(S) FOR DO/EO/US

COUTOS-THEVENOT, Pierre, HAIN, Ridiger, SCHREIER, Peter-Helmut, BOULAY, Michel

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

- . This is a FIRST submission of items concerning a filing under 35 U.S.C. 371
- 2. 

  This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. § 371.
- 3. This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
- 4. 🗵 A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
- 5. 🗵 A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. 🗵 is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. 

    has been transmitted by the International Bureau.
  - is not required, as the application was filed in the United States Receiving Office (RO/US).
- 6. A translation of the International Application into English (35 U.S.C. 371(c)(2)).
- 7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))

  - b.  $\square$  have been transmitted by the International Bureau.
  - c.  $\square$  have not been made; however, the time limit for making such amendments has NOT expired.
  - d. 
     \[
     \begin{aligned}
     & \text{have not been made and will not be made.}
     \]
- 8. A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3).
- 9. An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
- 10. A translation of the Annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

- 11. 

  An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
- 12. 

  An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
- 13. X A FIRST preliminary amendment.
  - ☐ A SECOND or SUBSEQUENT preliminary amendment.
- 14. 

  A substitute specification.
- 15. 

  A change of power of attorney and/or address letter

Copy of the International Preliminary Examination Report, with amended sheets and an English translation of the amended sheets; copy of the sequence listing on paper in French with an English translation thereof; copy of the sequence listing in computer-readable format (i.e., on diskette); copy of PCT/IB/308; copy of the International Search Report; copy of PCT/IB/306

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1990 M Street, N.W	7., Suite 800					
Washington, DC 20036-3425 Morris Liss NAME 24.510						
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# 534 Rec'd PCT/PTC 14 AUG 2000

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Pierre Coutos-Thevenot et al.

Serial No.: To be assigned

Art Unit: To be assigned

Filed: Herewith

Examiner: To be assigned

For: NUCLEIC ACID

Atty Docket: 0061/00091

**COMPRISING THE** 

SEQUENCE OF A STRESS-INDUCIBLE PROMOTER AND A SEQUENCE OF A GENE ENCODING A STILBENE SYNTHASE

### PRELIMINARY AMENDMENT

Commissioner for Patents Washington, D.C. 20231

Sir:

Prior to initial examination, please amend the above-captioned case as follows.

### IN THE CLAIMS

From the amended sheets, please amend claims 3, 6, 9, 12, 13, 20, 22, 23, 24, 25, and 27, and add new claim 29 as follows:

At claim 3, line 1, replace "one of Claims 1 and 2" with -- Claim 1--.

At claim 6, line 1, replace "one of Claims 1 to 5" with -- Claim 1--.

At claim 9, line 3, replace "one of Claims 1 to 8" with --Claim 1--.

At claim 12, lines 1-2, replace "one of Claims 9 to 10" with --Claim 9--.

At claim 13, lines 1-2, replace "one of Claims 9 to 12" with -- Claim 9--.

At claim 20, line 2, replace "one of Claims 9 to 19" with -- Claim 9--.

22. (Amended) Process for obtaining a cell according to [one of Claims 20 and 21, characterized in that] Claim 20, wherein a plant cell is transformed using a microbiological method including a system [or vector according to one of Claims 9 to 19] for expressing a stilbene synthase gene in plants comprising at least one nucleic acid.

At claim 23, line 4, replace "one of Claims 9 to 19" with --Claim 9--.

At claim 24, line 2, replace "one of Claims 9 to 19" with --Claim 9--.

At claim 25, lines 1-2, replace "one of Claims 20 to 21" with --Claim 20--.

At claim 26, line 2, replace "one of Claims 22 to 23" with --Claim 22--.

At claim 27, line 1, replace "one of Claims 24 to 26" with --Claim 24--.

--29. Process for obtaining a cell according to Claim 21, wherein a plant cell is transformed using a microbiological method including a vector for expressing a stilbene synthase gene in plants comprising at least one nucleic acid.--

#### REMARKS

The claims have been amended to eliminate multiple dependency and to improve their format. None of these amendments is believed to involve any new matter. Accordingly, it is respectfully requested that the foregoing amendments be entered, that the application as so amended receive an examination on the merits, and that the claims as now presented receive an early allowance.

Respectfully submitted,

Morris Liss, Reg. No. 24,510

Morris Lias

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### IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No.:

U.S. National Serial No.:

Filed:

PCT International Application No.:

PCT/FR99/00316

## **VERIFICATION OF A TRANSLATION**

#### I, Susan POTTS BA ACIS

Director to RWS Group plc, of Europa House, Marsham Way, Gerrards Cross, Buckinghamshire, England declare:

That the translator responsible for the attached translation is knowledgeable in the French language in which the below identified international application was filed, and that, to the best of RWS Group plc knowledge and belief, the English translation of the international application No. PCT/FR99/00316 is a true and complete translation of the above identified international application as filed.

I hereby declare that all the statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the patent application issued thereon.

Date: 17 July 2000

Signature of Director:

For and on behalf of RWS Group plc

Post Office Address:

Europa House, Marsham Way,

Gerrards Cross, Buckinghamshire,

England.

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NUCLEIC ACID COMPRISING THE SEQUENCE OF A STRESS-INDUCIBLE PROMOTER AND A SEQUENCE OF A GENE ENCODING A

STILBENE SYNTHASE

The present invention relates to plants which exhibit improved resistance to certain pathogenic agents which are sensitive to stilbenes, and relates, more specifically, to a set of constructs which combine a plant promoter which can be induced by a biotic stress, which stress is engendered, in particular, by the said pathogens, with (a) gene(s) encoding a stilbene synthase.

A large part of the world harvest of cultivated is regularly destroyed by parasites plants 15 pathogens. Among the possible options for decreasing or preventing the attack by these parasites on cultivated plants, chemical control (plant protection treatments) is the method which is most used. Nevertheless, the application of chemical products is not without conse-20 quences for the environment and sometimes presents technological problems as, for example, the appearance of new resistant pathogenic strains or, in the field of oenology, the difficulties which can arise inhibitors fermentation (the use οf of sterol biosynthesis can block yeast growth at the end of fermentation) or the presence of chemical products, such as procymidone, an anti-Botrytis product, which are sometimes found in wine.

The control method which consists in improving the resistance of cultivated plants to the diseases 30 which are caused by these pathogens has been envisaged as a way of overcoming the drawbacks associated with chemical control. It is possible, for example, in a first approach, to achieve this improvement by the 35 sexual route, i.e. using classical genetics, hybridizing the plants whose resistance is to be improved with tolerant varieties. Nevertheless, this approach is not always feasible (tolerant natural variety not known) or is not permitted by legislation such as, for example, in viticulture as a result of French legislation on Appellations d'Origine Controlée (A.O.C.) (registered designations of origin) which limits the grapevine varieties which are to be used for a given appellation (designation).

In a second approach, it is possible, using the modern techniques of cell and molecular biology, integrate, into the genome of the plant, one or more homologous or heterologous genes which make it possible to overexpress or express a molecule of interest, which protein nature, in order increase οf to production of a metabolite, or a metabolic pathway, or to open a new biosynthetic pathway or to synthesize a novel molecule for example for increasing the opening of 15 a new biosynthetic pathway, for example increasing the resistance of the plant by reinforcing its defence mechanisms with regard to the pathogens in question.

There are several different defence mechanisms of this type in plants. Some can be regarded as being 20 passive and are linked to the physicochemical characteristics of the cells, the epidermal tissues and/or the organs of the plant. Others belong to the dynamics of gene/gene interactions (plant resistance genes and pathogen avirulence genes, mechanisms of host/pathogen interactions). While these interactions lead to the development of a hypersensitivity reaction (rapid death of the cells of the plant around the point of infection in order to block colonization of the plant by the microorganism), they can also lead to the synthesis and accumulation of a whole series of 30 compounds. Of these, some can be parietal constituents which are involved in the formation of a "physical" barrier around the point of infection (callose, lignin, hydroxyproline-rich protein: HRGP, etc.), and other having 35 compounds can be molecules antimicrobial functions which are more or less well defined (phytopathogen-associated proteins: PR (pathogenesis-related proteins), etc.). The molecules of the phytoalexin type which synthesized are

accumulated by plants during, for example, host/pathogen interactions include, in particular the stilbenes, which are toxic, in particular for microorganisms. The term stilbene designates a group of chemical substances which 5 possess the trans-diphenyl-1,2-ethylene skeleton as the common basal structure, with resveratrol and pinosylvine being among the simplest. This basal skeleton synthesized in plants by a stilbene synthase or related enzymes from substrates such as malonyl-CoA, cinnamoyl-10 CoA or coumaroyl-Coa, which are substances which are present in all plants (flavonoid precursors). Genes for stilbene synthase or related enzymes have been isolated, sequenced and cloned, in particular from groundnut, orchid and grapevine. Using these genes, it has been 15 possible to transform plants such as potato, lucerne or tobacco, with these plants then exhibiting greater resistance than untransformed plants to pathogen attack EP-648839; MELCHIOR, F. et al., (EP-309862; Biochem. Biophys. 1991, 288, 2, 552-557; WIESE, W. et al., Plant Mol. Biol. 1994, 26,2,667-677; HAIN, R. et 20 al., Nature 1993, 361, 153-156).

The expression or overexpression of these molecules having antimicrobial functions can provide plants with a "natural" resistance in response to stresses, in particular stresses of the microbial type. However, constitutive overexpression of this type of protein necessarily has disadvantages for the plant (energy cost, slowing down of growth, etc.) (FISCHER, R. et al., The Plant Journal 1997, 11, 3, 489-498).

30 On the other hand, in some plants, such as grapevine or herbaceous plants, stilbenes are only found in some healthy tissues and at very low concentrations. Conversely following an infection or a lesion, these stilbenes increase strongly at the infected or damaged 35 site, since the stilbene synthase genes are inducible under conditions of biotic or abiotic stress (for example wounds, ultraviolet rays, etc.).

Nevertheless, this regulation is rarely present in plants of agricultural interest or, when it is

insufficiently effective. it can be For present, studies on phytoalexin synthesis the example, grapevine have demonstrated that the only healthy tissue in which stilbenes, including resveratrol, are present is healthy wood tissue. Stilbene is found to be present in the tissues of the grape berry when, on the one hand, the berry has been subjected to a stress such as attack by a pathogen (Botrytis cinerea for grey mould or Plasmopora viticola for grape downy mildew) and, on the other hand, only during the period up to the incipient ripening of the young fruit. By contrast, concentration of the stilbene decreases strongly from incipient ripening to maturation. However, damage due, for example, to Botrytis is rarely encountered during 15 the period up to incipient ripening but rather during the period close to maturation of the berry. For this reason, expression of the stilbene synthase gene has to be controlled with strong promoters which escape the natural regulation of the gene and which should be 20 inducible, in particular by the stress itself. The present invention specifically relates to a promoter of this nature.

The present invention relates to nucleic acids which comprise the sequence of the promoter for a lucerne PR protein linked to at least one sequence of a gene encoding a stilbene synthase.

The invention relates, in particular, to nucleic acids according to the invention, characterized in that the promoter for a lucerne PR protein is a promoter which can be induced in plants, in a tissue-specific manner or not, by a biotic or abiotic stress.

The invention also relates to nucleic acids according to the invention, characterized in that the sequence of the promoter for a lucerne PR protein is selected from the group comprising:

- a) the IND S1 sequence,
- b) any sequence corresponding to a fragment of the IND S1 sequence and having a promoter sequence effect in plants.

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The sequences of the promoter for a lucerne PR protein are preferred which exhibit at least 80% homology with the IND S1 sequence. Those sequences are particularly preferred which exhibit at least 90% or 95% homology with the said sequence.

The sequences of promoters for lucerne PR proteins according to the invention were obtained from regulatory sequences of genes for PR proteins by taking advantage of the incompatibility response (hypersensitivity reaction, HR) obtained in the host/parasite relationship between lucerne (Medicago sativa) and Pseudomonas syringae pv pisi for the purpose of isolating the regulatory sequences of genes which are responsible for this reaction.

When Pseudomonas attacks lucerne, the occurrence of a plant reaction is observed in the region adjacent to the necrosis caused by the bacterial infection.

Plant material was therefore removed following the bacterial attack in order to construct a cDNA library from the messenger RNAs which were produced in the regions adjacent to the necrosis. Amplification by polymerase chain reaction (PCR), using polynucleotides corresponding to motifs which conserved in leguminous PR protein genes, enabled a radioactive probe to be obtained which was then used to select transcripts in the cDNA library. One of these after sequencing, (cDNA-PR7) was adopted since, exhibited good homology with equivalent genes encoding PR proteins and known from other plants (cf. Figures 1 and la, depicting the general scheme of the method for isolating the promoter).

Analysis showed that it corresponded to a gene encoding a class 10 PR protein according to the VAN LOON (1994) classification. This gene was therefore designated Ms PR10-1 (Medicago sativa PR class 10 protein, clone 1). The isolated and cloned cDNA PR7 made it possible to obtain two probes due to the presence of an internal Bam HI site (B in Figure 1). These probes, EB and BE (E corresponding to the Eco RI site in Figure

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1) named respectively 5' and 3', were used to screen a lucerne genomic library. Among the clones obtained which were recognised by the 5' and 3' probes, one of them, C15, was selected and sequenced (6.1 Kb). It itself also 5 possesses, logically, a Bam HI site which made possible to obtain two novel EB and BE fragments of 2.4 kb and 3.7 kb, named respectively E-B(g) and B-E(g), g indicating the genomic nature of the fragments obtained (see Figure 1a). Fragment E-B(g), which is located in 5' 10 of the C15 clone, and which comprises the promoter and a portion of its Ms PR10-1 gene coding sequence, was inserted into the Eco RI and Bam HI sites of the bluescript plasmid. The plasmid was linearized by means of a Pst I site which is located upstream of Bam HI in fragment E-B(g). A deletion from 3' to 5' was carried out on this fragment until the IND S1 promoter sequence was obtained (Figure 3). A blunt end ligation made it possible to reposition another Bam HI site, internal to the C15 clone, at the end of the promoter sequence of Under these conditions, 13 PR10-1 gene. nucleotides of the coding sequence of the Ms PR10-1 gene, located upstream of this Bam HI site which is internal to the C15 clone, were thus integrated into the IND S1 promoter sequence: the whole can be isolated with 25 an Eco RI/Bam HI digestion (see Example 1).

In the description, PMs PR 10-1 is also intended to mean any nucleic acid fragment of the IND S1 sequence with a promoter effect in plants and the IND S1 sequence with 13 nucleotides of the coding sequence of the Ms 30 PR10-1 gene as described below.

The invention also relates to nucleic acids according to the invention, characterized in that the sequence of a gene encoding a stilbene synthase, whether homologous or heterologous, is selected from the genes isolated from groundnut, orchid, grapevine and pine genomes (EP-309 862, EP-464 461).

Of the said nucleic acids, preference is given to the nucleic acids which encode a grapevine stilbene synthase, in particular those described in the article

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by HAIN, R. et al., Natrue 1993, 361, 153-156 and in that by WIESE, W. et al., Plant Mol. Biol., 1994, 26, 2, 667-677; the nucleic acid corresponding to the sequence *vst1* of the said articles is that which is most preferred.

The nucleic acids enabling the stilbene synthase gene(s) to be expressed will naturally be able to include, in particular, apart from the said gene(s), polyadenylation sequences at the 3' end of the coding strand and enhancer sequences from the said gene or from a different gene.

Naturally, the nucleic acid sequences will have to be adapted in order to ensure that the gene is actually read in the correct reading frame with the promoter and it will obviously be possible to foresee using, if necessary, several promoters of the same type as well as several enhancer sequences.

It is also possible to use the nucleic acids according to the present invention to express several stilbene synthase genes, either arranged in tandem or carried by different expression systems.

The nucleic acids according to the invention can be used to create expression systems in plants, which systems can be inducible and/or constitutive depending on the tissues or organs of the plant which are transformed (cf. Examples 2, 3 and 4).

The present invention therefore also relates to systems for expressing at least one stilbene synthase gene in plants, characterized in that they comprise at least one nucleic acid according to the invention. Of the systems according to the invention, preference is transformation to vectors, particularly transformation vectors of the plasmid Advantageously, the said transformation vectors characterized in that they can be transferred into Agrobacterium strains.

The stilbene synthase genes which are able to be expressed by the nucleic acids according to the present invention are placed under the control of the PMs PR10-1

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promoter for the purpose of activating, in plants, mechanisms of resistance to pathogens which are sensitive to stilbenes, in particular to resveratrol, to pinosyl grapevine or to their glycosylated derivatives such as picein or to oligomers such as the viniferins. Parasites of this nature which are sensitive to stilbenes and which may be mentioned are Botrytis cinerea, Plasmopora viticola, Eutypa lata, etc.

Preference is given to those expression systems
10 according to the invention which are characterized in
that they can be induced in plants by a biotic or
abiotic stress.

From the said biotic stresses according to the invention preference is given in particular to biotic stresses which are engendered by the attack of a parasite which is sensitive to stilbenes, such as a virus, a bacterium, a yeast, a fungus, in particular Botrytis cinerea or Plasmopora viticola.

From the said abiotic stresses according to the invention, preference is given in particular to abiotic stresses engendered by a mechanical wound such as that caused in particular by an insect or by a physical phenomenon such as wind or frost.

The present invention also relates to plant 25 cells which are transformed with a system or a vector according to the invention. Advantageously, the said plant cells are grapevine cells.

The present invention also relates to processes for transforming plant cells using a microbiological 30 method, including the systems or vectors according to the present invention.

The invention furthermore relates to processes for obtaining plants which express one (or several) stilbene synthase gene(s), characterized in that cells of the said plants are transformed using a system or a vector according to the invention, the cells expressing the said gene(s) are selected and a plant is regenerated from these cells.

The most frequently used transformation methods

which may be mentioned are, in particular, the methods which employ Agrobacterium, whether this be Agrobacterium tumefaciens or Agrobacterium rhizogenes, biolistic or any other techniques (electroporation, 5 etc.).

These methods are known (REAM, W., 1989; NEGRETIU, I. and GHARTI-CHHETRI, G.B., 1991; CASSE-DELBART, F., 1996; STANFORD, J.C., 1990) and will not be described again in detail.

The technology, which in particular makes use of plasmid systems, enables a first transformation of a competent bacterial straim, in general *E. coli*, to be effected, which transformation enables the structure of the plasmids to be cloned and monitored. The strain is then used to transfer the recombinant plasmids into agrobacterial strains, which will then be used to transform the plant cells.

The plants comprising an expression system or cells according to the invention are part of the inven-

The plants which are obtained by implementing the processes according to the invention are also part of the invention.

Finally, the invention relates to plants accor-25 ding to the invention, characterized in that the plants are plants of agricultural interest, in particular grapevine plants.

Other characteristics and advantages of the constructs and the processes according to the present invention will be evident from the examples which follow.

#### Figure legends

35 Figures 1 and 1a: General scheme depicting the different steps in the method for isolating the inducible promoter PMs PR10-1, corresponding to the IND S1 sequence.

Figure 2: Representation of the various clones which were isolated and which correspond to the Southern blot,

which blot was hybridized with the 5' and 3' parts of cDNA-PR7, which parts were delimited by an internal Bam HI (B) site which was detected in this cDNA.

Restriction sites: E = Eco RI, B = Bam HI.

- 5 The values indicated in the figure are expressed in kb (kilo bases).
  - Figure 3: DNA sequence corresponding to the IND S1 sequence, which is the isolated genomic sequence of the inducible lucerne promoter PMs PR10-1.
- 10 Figure 4: DNA sequence corresponding to the sequence of a gene for grapevine stilbene synthase which is modified by adding an adaptor (modified *vst1*).

The modified part is depicted in italics.

The coding and noncoding (intron) parts are 15 depicted in upper case and lower case letters, respectively.

Figure 5: DNA sequence comprising the sequence of the inducible promoter PMs PR10-1 (corresponding to the IND S1 sequence in lower case) linked to the sequence of a gene for grapevine stillbene synthase which is modified by adding an adaptor (modified vst1, corresponding to Figure 4). Between the two (framed in the sequence) are (end of the promoter in lower case and start of the gene

containing the translation start codon in upper case)

- the 13 nucleotides which come from an internal sequence of the coding frame of the Ms PR10-1 gene; since the ATG has been positioned in reading frame with the modified vst1 gene, these nucleotides are thus integrated into the coding frame of vst1.
- The sequence of the inducible promoter PMs PR10-1 includes the 7 of the 13 nucleotides of the sequence of the gene Ms PR10-1 (in lower case in the frame).

The coding and noncoding (intron) parts of the 35 part corresponding to the sequence of the gene for grapevine stilbene synthase are depicted in upper case and lower case letters, respectively.

<u>Figure 6</u>: Demonstration of the induction of the gene encoding a stilbene synthase with UV light.

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The RNAs are extracted from approximately 1 g of leaves 17 hours after inducing with UV light. From 10 to 20 µg are loaded onto a formaldehyde/formamide denaturing gel. After migration (3 V.cm<sup>-1</sup>), the RNAs are transferred to a nylon membrane and fixed by exposure to UV light (254 nm, 33 mJ.cm<sup>-2</sup>). The Northern blot is obtained by hybridizing, at 65°C and overnight, with the biotinylated probe vst1.

The explant which is used as the starting 10 material consists of leaves which have been isolated from 41B (control) vitroplants or 41B vitroplants which have been genetically transformed with a construct (13 kb insert comprising two complete stilbene synthase genes, vst1 and vst2, a fragment of grapevine genomic 15 DNA and another truncated grapevine stilbene synthase gene (vst3)) which integrates supernumary copies of genes encoding grapevine stilbene synthase under the control of their own promoters (clones 2 and 3 corresponding to clones 55-2 and 55-3, respectively). 41B: hybrid V. vinifera, Chasselas Η 20 stock-vine V. berlandierii.

Figure 7: Kinetics of stilbene synthase mRNA accumulation following induction with UV light.

The RNAs are extracted from approximately 1 g of leaves. From 10 to 20 µg are loaded onto a formaldehyde/formamide denaturing gel. After migration (3 V.cm<sup>-1</sup>), the RNAs are transferred to a nylon membrane and fixed by exposure to UV light (254 nm, 33 mJ.cm<sup>-2</sup>). The Northern blot is obtained by hybridizing, at 65°C and overnight, with the biotinylated probe vst1.

The explants are leaves which have been isolated from 41B vitroplants. The control is a clone which has not been transformed genetically, contrary to clones 2 and 3 (corresponding to clones 55-2 and 55-3, respectively), which have integrated, into their genome, the 13 kb insert (see above) containing genes encoding grapevine stilbene synthases (vst1 + vst2). 41B: stock-vine hybrid V. vinifera, Chasselas H V. berlandierii. Figure 8: Quantity of resveratrol present in vitroplant

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leaves which have been treated with UV light for 8 min and analysed at different periods after induction.

The quantities are expressed in  $\mu g$  per g of fresh material.

5 NI: not induced

The control consists of leaves taken from untransformed 41B. PCT 55-2 and 55-3 are two transformants which have integrated the 13 kb insert comprising two complete stilbene synthase genes (vst1 + 10 vst2).

Figure 9: Inhibition of the growth of Botrytis cinerea mycelium, strain 916T, after 7 days at 20°C.

The mycelium is cultured on a malt/glucose medium containing different concentrations of 15 resveratrol.

Figure 10: Photographic plate 1

Macroscopic observations which are characteristic of different varieties of grapevine in interaction with *Botrytis cinerea* 5 days after 20 inoculating vitroplant leaves with a conidial suspension - untransformed plants.

Upper row:

- Left: Folle blanche variety, clone 280, susceptible.
- Right: Pinot noir variety, clone 386, moderately tolerant.

Lower row:

- Left: Ugni-blanc variety, clone 479, tolerant.
- Right: 41B stock-vine, tolerant.
- 30 Figure 11: Photographic plate 2

Macroscopic observations which are characteristic of 41B stock-vine clones which have been transformed with different constructs (145: Ms PR10-1 promoter - vst1 gene; 55: 13kb insert comprising two genes, vst1 and vst2, under the control of their own promoters), in interaction with Botrytis cinerea 5 days after inoculating vitroplant leaves with a conidial suspension.

Upper row:

35

- Left: Clone 145-2.
- Right: Clone 145-5.

#### Lower row:

- Left: Clone 145-6.
- 5 Right: Clone 55-3.

### Figure 12: Photographic plate 3

Fluorescence microscopy observations which are characteristic of different grapevine varieties in interaction with *Botrytis cinerea* 5 days after 10 inoculating vitroplant leaves with a conidial suspension.

Fluorescence: filter unit A (excitation of from 340 to 380 nm; stop filter at 425 nm). Resveratrol gives out a bluish white and blue (depending on its concentration) fluorescence light while chlorophyll fluoresces red. The black region corresponds to the region of fungal infection or to necrotic regions when they are small.

#### Upper row:

- 20 Left: Folle blanche variety: clone 280, susceptible, little or no synthesis of resveratrol.
  - Right: Pinot noir variety, clone 386, moderately tolerant. Synthesis of resveratrol in the veins and in the region of fungal maceration.
- 25 Lower row:
  - Left: Untransformed stock-vine 41B (tolerant). Synthesis of resveratrol intense in the veins and in the lamina around the small and very localized regions of infection (necrotic regions).
- Right: Stock-vine 41B transformed with construct 145, i.e. the *vst1* gene promoter PMs PR10-1, clone 145-5, very tolerant. Strong synthesis of resveratrol around and in the region of infection, in veins and over almost the whole of the lamina of the 35 leaf.

Example 1

Obtaining genomic clones which comprise regulatory sequences of lucerne PR protein genes

# A) Obtaining a probe for the purpose of finding the promoters (cf. Figures 1 and 1a)

The incompatibility response (hypersensitivity reaction, HR) which is obtained in the lucerne (Medicago sativa) and Pseudomonas syringae pv pisi host/parasite 10 relationship made it possible to construct a cDNA library. It was prepared from messenger RNAs which were extracted and purified from the zone adjacent to the necrosis caused by the bacterial infection. Plant material was taken 6 hours after infiltrating with the bacterial suspension.

The PR proteins in leguminous plants are known to have motifs which are conserved; it was therefore possible to synthesize oligonucleotides corresponding to these motifs defined on the basis of sequencing which 20 had already been carried out on pea and soya bean PR proteins. PCR amplification enabled a radioactive probe to be obtained which was then used to select transcripts in the cDNA library. One of these clones, cDNA-PR7, was adopted since after sequencing it exhibited 87% homology 25 with the genes encoding the pea and soya bean PR proteins. Analysis showed that it in fact corresponded to a gene encoding a class 10 PR protein according to the VAN LOON et al. (1994) classification. It was PR10-1 (Medicago sativa PR class 10 designated Ms protein, clone 1).

A control which was carried out on lucerne by means of Northern blotting showed that, in the incompatibility reaction, the corresponding transcript began to accumulate 3 hours after infection, passed through a maximum between 24 and 48 hours and decreased slowly from 72 hours onwards.

This fragment is characterized by the existence of an internal Bam HI site (marked with a B in Figure 2) which delimits two moieties:

- the one, termed 5' and of approximately 340 bases, includes the region upstream of the ATG (which is transcribed but not translated) and a downstream sequence corresponding to 306 bases,
- 5 the other, termed 3', corresponds to the end of the coding moiety, i.e. 165 bases, and to the untranslated 3' region, that is 186 nucleotides from the stop codon to the beginning of the pcly A.

# 10 B) Isolation of genomic clones comprising promoters of PR proteins

1) Isolation of genomic clones

A lucerne genomic library, prepared in EMBL4 (titre:  $7.10^8$  p.f.u. (plate-forming units)  $\times$  ml<sup>-1</sup>), was 15 used on this occasion and 6.10<sup>5</sup> p.f.u. were plated out. The 5' fragment of Ms PR10-1 (cDNA PR7) was used as a probe to screen this library and 45 clones gave a signal, which was strong for 13 of the clones. Restriction mapping and hybridization which were carried 20 out using the 5' and 3' fragments of Ms PR10-1 led to the conclusion that there were 7 distinct clones (cf. Figure 2). Comparison of the sizes of the 7 clones (9.3; 6.5; 6.1; 5.8; 4.8; 4.2; 2.2 kbp) which were obtained by screening the library with those of bands detected on a 25 blot of lucerne genomic DNA showed good agreement between these two types of experimental data (cf. Figure 2). It was therefore possible to deduce from this that the genes encoding the PR7 protein corresponded to a small multigenic family.

The fragments (EcoRI/EcoRI sites) of these clones (apart from clone C12) were then subcloned in whole or in part and sequencing was undertaken.

#### 2) Sequencing carried out

One clone was chosen for sequencing first, i.e. clone C15 (cf. Figures 1 and 1a).

The initial sequencing work demonstrated the presence of an intron of approximately 315 nucleotides in the open reading frame of the gene encoding the PR

protein. The clone under study was then analysed after having been digested with  ${\it Bam\ HI}$  (cf. Figure 1a).

Clone C15: 6.1 kb

Analysis of the clone with Eco RI and Bam HI enabled two fragments to be obtained, i.e. E-B (approximately 2.4 kbp) and B-E (approximately 3.7 kbp). After sequencing and comparing the coding sequence (interrupted by an intron of 600 nucleotides) with that of Ms PR10-1 (cDNA-PR7), it appeared that this genomic clone was absolutely identical to this reference cDNA.

3) Analysis of the expression of the isolated clones in the lucerne/Pseudomonas system

The experiments focused on clone C15, using the 5' extension technique in order to determine the messenger molecules which were in fact transcribed during induction of the defence reactions. This technique has the additional advantage of making it possible to locate the transcription initiation site. The results demonstrated that clone C15 was in fact expressed during induction of the defence reactions in the leaves as part of the lucerne/Pseudomonas interaction.

#### Example 2

5 Genetic transformations which were carried out in order to verify the promoter activity of the isolated clones

### A) Promoter regions used

Two promoters were used for these verificatory transformations: a control promoter and the PR promoter which was isolated from the lucerne genome and which was derived from C15 (corresponding to the PMs PR10-1 promoter).

#### 35 1) CaMV-35S promoter

This promoter, which is constitutive, is used as a standard promoter. It corresponds to the sequence for regulating the transcription of the gene for the 35S RNA subunit of the cauliflower mosaic virus (CaMV). The

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promoter region which was used to effect the construct with the gus reporter gene in fact corresponds to a fraction of this promoter, which fraction was reisolated in the form of an EcoRI/BamHI fragment from the plasmid pDH51 (PETRZAK et al., 1986).

#### 2) PR promoter

A study was carried out of the promoter regions of the C15 genomic clone. This promoter was subsequently termed PMs PR10-1.

#### PMs PR10-1:

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derived from the *EcoRI/BamHI* Ιt is fragment of 2.4 kb of the C15 clone (Figure 1a). The integration of this fragment into the binary plasmid 15 upstream of the reporter gene (see below) was made difficult by the fact that there was no restriction site in clone C15 between the TATA box (initiation of transcription) and the ATG (initiation of translation). Deletion experiments were therefore carried out until a 20 fragment of approximately 1.5 kb was obtained (sequence IND S1). A Bam HI site was then added, by blunt-end ligation, to the resulting fragment in order to enable it to be inserted upstream of the different coding sequences which were subsequently used. This fragment therefore comprises, expressed by reference to the cDNA which was used to clone it and also the upstream promoter region: 39 terminal nucleotides of the 5'UTR (UnTranslated Region) of the Ms PR 10-1 gene, located 10 bp from the initiating ATG codon, the ATG of the Ms PR10-1 gene and a short fragment of its coding region (10 bp) immediately upstream of the integrated Bam HI the cloning sites, site. Taking into account constructed in this way has promoter which is potential ATG, which could lead to the presence of two 35 ATG codons at a short distance from each other when constructing chimeric genes. There could then be a risk of altering the coding frame of the gene which is used (reporter gene or stilbene synthase gene).

For the transformation experiments with the

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reporter gene, PMs-PR10-1 (PRI) was used as such after having been cloned into the STRATAGENE Bluescript pSK+/-plasmid. It was possible to reisolate it in the form of an approximately 1.5 kb *EcoRI/BamHI* fragment.

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- 3) Plasmids used
- a) p35S gus intron (VANCANNEYT et al. 1990)

This plasmid is a derivative of pBin19 (BEVAN, 1984) and as such possesses the right and left borders of the binary plasmids, enabling the segment contained between these borders to be inserted into plants using agrobacteria.

The development of the gus intron (intron derived from the potato LSI gene) reporter gene made it possible to eliminate the false positives (in particular during transient expression) which were due to contaminating agrobacteria. These bacteria are not able to splice the introns.

In the standard system, this gene, which encodes a  $\beta$ -glucuronidase, enables a blue coloration to be obtained when a specific substrate (5-bromo-4-chloro-3-indolyl- $\beta$ -glucuronide) is used. This blue colour then indicates that the analysed plant has been transformed and, as a consequence, that the coding sequence of the gene corresponding to the enzyme has been transcribed and, therefore, that the promoter which controls it has been induced.

b) pFR97

This plasmid was constructed by one of the laboratories participating in the project for testing promoter efficiency (P. RATET, ISV, cited in SZABADOS et al., 1995). It has, in particular, the advantage of possessing a multiple cloning site, making it possible to effect transcriptional fusion with the coding frame of the gus gene (E. coli uid A) containing the LSI intron. It also possesses some of the characteristics of the preceding plasmid p 35S gus intron (borders for integration into the plant genome, selection gene affording resistance to antibiotics of the neomycin

type: npt II gene).

The activity of the promoter can be visualized and measured by histochemical and enzymic tests of the GUS type.

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#### 4) Derivatives of pPR97

Two main constructs were made and subsequently used for transforming model plants.

#### a) pR97 - 35S

The 35S promoter, cloned into plasmid pDH51 (cf. paragraph 5 below), was excised and inserted into the multiple cloning site of pPR97, upstream of the reporter gene, in the form of an EcoRI/BamHI fragment. This plasmid is both a positive control, to demonstrate that the construct functions, and a reference since the 35S promoter was placed in the same environment as the promoter isolated from the PR protein clones.

#### b) pPR97-PMs PR10-1

The 1.5 kb fragment was inserted into the same 20 cloning site as that defined above, in this case too in the form of an *EcoRI/BamH1* fragment.

#### c) pG3-3

This enabled a strong positive control for the histochemical and enzymic tests to be obtained by cloning two 35S promoters as an inverted tandem. The activator sequences of the promoters then act synergistically. The coding frame of the gus intron gene was then placed under the control of one of the two 35S promoters.

#### 30 5) Preparation of Agrobacterium strains

The different plasmids were used to transform competent E. coli strain DH5 $\alpha$  bacteria by thermal shock in a calcium chloride medium. After selecting transformed bacteria on a medium containing antibiotic (kanamycin) and using a miniprep to check that they were recombinant, these bacteria were then used to transfer the recombinant plasmids into Agrobacterium strains by means of triparental conjugation using the E. coli strain HB101 harbouring the autotransferable plasmid

pRK2013 (DITTA et al., 1985).

Two agrobacterium strains were used: EHA 105, a disarmed Agrobacterium tumefaciens, used for regenerating transformed plants (stable transformations) and A4TC24 Agrobacterium rhizogenes, which was used to obtain the hairy root reaction and composite plants whose roots are transformed but which otherwise have a phenotype which is identical to the original phenotype.

10 6) Genetic transformations on model plants

Two types of transformation (transient and stable) were carried out using three model plants, i.e. Nicotiana benthamiana, Medicago truncatula and Lotus corniculatus.

The results presented will in the main be those obtained with *N. benthamiana*.

#### 7) Transient transformations

This first series of experiments was carried out 20 for the purpose of rapidly verifying that the constructs which were produced using the *gus* gene functioned in the eukaryotic cells.

N. benthamiana leaves were therefore excised and cocultured on an agar medium together with the different derivatives of the EHA 105 strain. Histochemical tests were then performed 48 h after the transformation and subsequently examined after incubating overnight (12 h).

The control plasmids, p35S gus intron and pPR97-35S, gave a positive GUS coloration even if this was weak in the case of the second plasmid.

This weak reactivity is without doubt due to a construction problem since a part of the polylinker had to be retained in the vicinity of the transcription initiation site and the ATG of the gus gene. Since this polylinker part encompasses a repeat sequence it can interfere with transcription of the gene.

The pPR97-PMs PR10-1 construct gave a gus gene activity which was similar to that of the 35S gus intron positive control. This promoter, which was expected to

be inducible, therefore exhibited an effect which was comparable to that of a constitutive promoter.

This result can be explained as being the consequence either of the bacterial infection or the injuries 5 inflicted on the leaves during sampling or during the first hypothesis cannot Since culturing. verified, the experimental protocol was modified in order to decrease the stress caused to the explants (increase in the osmolarity of the coculture medium 10 using sucrose concentrations of from 10 to 30  $\mathrm{g.1}^{-1}$ , applying a relatively high vacuum range: a relative vacuum of from 10 to 80 mm of mercury and the production of relatively severe lesions on the leaves accompanied by significant or moderate crushing of the epidermis).

The results demonstrated that the number of transformed cells increased as the pressure increased. However, a compromise should be found in order to achieve stable transformations since the large number of transient transformations which are obtained in this 20 case frequently turn out subsequently to be lethal for the cells. The latter are then unable to give rise to cali and thus regenerate shoots and then plants. In any case, the inducible nature of the promoter is confirmed in part since, while the coloration due to the 35S-gusintron construct can be detected up to 5 days after coculturing, that obtained using pPR97 - PMs PR10-1-gus intron appears more rapidly at 48 hours but then subsides very rapidly.

- 8) Stable transformations 30
  - a) Tobacco: N. benthamiana

A transformation series was carried out using pPR97-35S, pPR97-PMs PR10-1 and pG3-3. A substantial number of plantlets was obtained for this transformation 35 series. An attempt was made to obtain at least 7 acclimatized plants for each of the plasmids used. However, this was not possible for p35S-gus intron, where only 5 plants were regenerated and acclimatized. The results which were obtained are compiled in Table 1.

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Table 1: Stable transformations obtained in N. benthamiana by transforming with the Agrobacterium tumefaciens strain EH 105 and its derivatives.

Constructs	Cali/expl ants cul- tured	Shoots obtained	Plantlets in vivo Accl.	
p35S-gus intron	10(45)	6(1)	5	5
pPR97-35S gus intron	115/122	23	13	12
pPR97-PMs PR10-1 gus intron	135/139	27	20	7
pG3-3-35S as an	not			
(strong promot.)	assessed	37	28	20

## Legend to Table 1:

The results are expressed as the quantity obtained.

Accl.: Acclimatized plantlets

Number of shoots per explant: The figure in brackets corresponds to the number of shoots which were obtained at one month in the case of the first series. In this series (comprising the 35S gus intron construct), the cali were left longer on the culture medium in order to obtain a maximum number of shoots and therefore of plants to acclimatize. In the case of the second series, a sufficient number of shoots had been obtained after one month and the experiment was then stopped.

Genetic transformations: These were done in a standard

manner on 1 cm<sup>2</sup> pieces of leaf lamina, which pieces were immersed in the *Agrobacterium* suspension for 30 seconds and then cocultured for 48 hours before being subcultured onto agar medium for cell division and caulogenesis (MURASHIGE et al., 1962), 0.1 mg.1<sup>-1</sup> NAA (naphthaleneacetic acid), 1 mg.1<sup>-1</sup> BAP

25 (benzylaminopurine), 400 mg.1<sup>-1</sup> cefotaxime (elimination of the agrobacteria) and 70 mg.1<sup>-1</sup> kanamycin (agent for selecting transformed cells). Once the first shoots have

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appeared (approximately one month after coculturing), they are placed on a rooting medium which is identical to the former medium except that it does not contain plant hormones.

Rapid analysis of the results in terms of the expression of the gus intron gene depending on the nature of the promoter which is situated upstream of the coding frame of the gene shows that the PMs PR10-1 promoter gives the best results of all the promoters 10 tested. A more detailed analysis is presented below.

## 9) Characteristics of the PMs PR10-1 promoter Plasmid pPR97-PMs PR10-1-gus intron

This promoter gave the best results with differ-15 ences in the constitutive expression of the gus gene depending on the organs tested.

### a) Activity in cali

Strong constitutive expression was found. A few minutes of incubation were sufficient to obtain a posi-20 tive histochemical test. The promoter is therefore strongly induced in this type of material, something which is in agreement with the results obtained by VAN LOON (1985). Cali which are cultured in vitro are in a state of stress and the PR proteins are expressed under these conditions.

## b) Activity in acclimatized whole plants Roots

The promoter is induced and the histochemical test is positive after 2 hours of incubation (as against 30 5 hours when the 35S promoter is used). The activity of the gus gene is not uniform in tobacco roots; only the epidermis of the old parts and the apical meristem gave the blue coloration which is characteristic for the test. According to the literature, defence gene activity in the roots is also observed under conventional conditions.

#### Flowers

A strong constitutive activity was found in the

flowers and, more particularly, in the anthers and the pollen, of all the tobacco plants which were transformed with this construct. Gus gene activity was also detected in the trichomes of the sepals and, more weakly, in the petals. These results are also in agreement with those of VAN LOON (1985), which indicate that defence genes are induced in the floral parts.

#### Leaves

Weak constitutive gus activity was observed in the trichomes of young leaves of adult plants. In the case of tobacco, the rosette stage with large leaves corresponds to the juvenile stage and the ageing stage to that of the formation of seeds. This weak activity was predominantly observed in the multicellular trichomes. To our knowledge, the expression of a PR protein in such structures has never been described.

While it is possible, therefore, that the isolated PMs PR10-1 promoter has a constitutive inducing activity in the trichomes of tobacco leaves (3 plants out of 7), this activity appears to be under the influence of the development stages.

In the absence of induction by a pathogen, the activity of the promoter in tobacco is therefore limited to the root, to floral parts (anthers and pollen) and to a few cells of the aerial part (basically trichomes).

# Example 3 Other plant species which were transformed

#### 30 1) Medicago trunculata

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In the case of this species, an attempt was made to create composite plants, that is plants which possess, at one and the same time, a wild-type aerial part (not genetically transformed) and transformed roots.

Young germinations were used. After development of the main root, the excised hypocotyles were soaked in a suspension of *Agrobacterium tumefaciens* EHA 105 harbouring either plasmid p35S-gus intron or plasmid

pPR97-PMs PR10-1-gus intron so as subsequently to obtain newly formed roots which were transformed. After one week, roots were obtained and a histochemical GUS test was carried out. For this experiment, the control consisted of young germinations which were treated in an identical manner to the previous batches (hypocotyles were excised, but were not soaked in the agrobacterial suspension).

While all the explants of the control batch formed new roots within one week, only 50% of the explants reacted, by contrast, in the case of the batches which were treated with the agrobacteria. Whatever the treatment, no root gave a positive response to the GUS test. On the other hand, even though necrosed, the base of the hypocotyles in the treated batches often reacted by giving a blue coloration (presence of tranformed cells). The necrosed part of the explants was then excised and they were set to rooting again. 50% then developed newly formed roots, some of which were to be positive to the test in a few regions.

containing both (roots Chimeric roots untransformed cells) were transformed cells and transformed obtained, wi.th the parts therefore corresponding to cell lines which had integrated the construct into a basal stem cell.

The two constructs which were tested, i.e. p35S-gus intron and pPR97-PMs PR10-1 gus intron, gave these transformed root cell lines in 3 and 2 explants, respectively, out of the 6 which were subjected to experiment in the case of each batch.

Although the experimental model was not adapted to the topic under investigation (study of the expression of PR proteins in association with the phenomenon of nodulation by *Rhizobium*), it nevertheless demonstrated that the PMs PR10-1 promoter is just as functional in this plant as in the original plant (*Medicago sativa*).

#### 2) Lotus corniculatus

In this case too, the experiment had the aim of studying the induction of the promoter in association with nodulation by the symbiotic bacterium Rhizobium 5 meliloti NZP 2037 (PETIT et al., 1987). Composite plants were therefore produced by transforming cells of the germinations with Lotier young hypocotyl of Agrobacterium rhizogenes strain A4TC24 in order obtain the hairy root phenomenon (hairy root phenotype). 10 Once this had developed, the main roots were excised and the plantlets were placed in a liquid medium in order to amplify the development of the phenomenon. Once the plants were acclimatized, induction of the promoters in association with nitrogen fixation symbiosis was studied 15 by putting the plantlets under nodulation conditions (BLONDON, 1964). The two promoters which were used for the study were the same as those which were used in the experiment carried out with M. trunculata: 35S and PMs PR10-1. Using these two constructs, less than 10% of the 20 roots having a hairy root phenotype exhibited roots which were positive to the GUS test. Generally speaking, the roots having this phenotype gave fewer nodules than did the control roots.

In the case of those which were obtained with the construct comprising the 35S promoter, it was only the nodules which gave a positive response to the GUS test, while, in the case of the other construct (PMs PR10-1 promoter), the coloration developed over the whole of the root apart from the secondary root initiation point. Apart from that, this latter construct did not enable nodules to be obtained on the roots derived from hairy root in interaction with Rhizobium meliloti.

#### Example 4

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Study of the hypersensitivity reaction of tobacco which is transformed with the constructs using promoters of the lucerne PR gene and the gus intron gene

A) Hypersensitivity reaction in N. benthamiana which is transformed with the constructs linking the lucerne PR gene promoter and the gus intron gene

10 1) Hypersensitivity reaction (HR) test

The hypersensitivity reaction (HR), developed in the N. benthamiana/Pseudomonas syringae pv. pisi interaction, was used in this study. Transformed tobacco plants, which had incorporated the different inserts of plasmids p35S gus intron and pPR97- PMs PR10-1-gus

intron into their genome, were acclimatized and then infiltrated with a suspension of *P. syringae* (ESNAULT et al., 1993) at a concentration of 10<sup>9</sup> bacteria per ml. The solution was injected into the lamina using a

hypodermic syringe. Using a model of this nature, the HR reaction is regarded as being well developed after 48 hours. Leaves which had been infiltrated with the bacterial suspensions were removed at 24, 48 and 96

hours after inoculation in order to assess induction of 25 the different promoters under study using the GUS histochemical test. The same histochemical test was also used on the leaves situated below the infiltrated leaf in order to assess any possible systemic response.

- 30 2) Study of the induction of the promoters under the HR reaction conditions
  - a) 35S constitutive promoter

In the case of the 35S constitutive promoter (plasmid pG3-3, for example), the inoculation with 35 P. syringae did not modify the response to the test, with this response being evident after a few minutes of incubation. Therefore, infiltration with the bacteria does not alter the constitutive glucuronidase activity which is obtained with the 35S promoter.

b) Promoters of PR protein genes: Ms PR10-1 promoter

As shown in Table 2, this promoter is readily inducible by pathogen attack. While the HR reaction is still not completely developed at 24 hours (48 hours for the effect to be fully displayed), the GUS test is already positive. In the case of the young transformed tobacco plants which were obtained, the coloration is weak and predominantly produced in the lamina of the infiltrated leaf.

10 With regard to the systemic response, i.e. the response in the leaf below the infected leaf, the coloration is only present in the lamina. The adult (having developed stems but still not having flowered) and juvenile (rosetted) tobacco plants have the same 15 type of response, with a weak gus gene activity as determined by the histochemical test.

In the case of the older tobacco plants, either in flower or carrying seeds, the coloration which is obtained in the test is more intense, especially in the veins and the trichomes of the infected leaf, with coloration only being present in these tissues in the case of the systemic response.

These observations therefore demonstrate differences in the expression of the reporter gene depending on the age of the plant. This response, which is dependent on the developmental stage of the plant, was found in most of the studies carried out on plant PR proteins. Induction of the PMs PR10-1 promoter is a transient phenomenon since expression of the reporter gene is no longer evident at 96 hours after inoculating the bacteria.

The induction is not limited, either, to the HR reaction which is obtained in the plant/bacterium interaction. The same type of response was obtained with the PMs PR10-1-gus intron construct when one of the explants was infected with a fungus. Homogeneous expression of the gus gene was then evident over the entire infected leaf apart from the contaminated region, which was necrosed.

Table 2: Induction of the different promoters under study in association with the HR reaction between N. benthamiana and P. syringae.

Construct	24 h af inoculat	<del></del>	96 h after inoculation		
	Infiltrated leaf	Leaf below	Infiltrated leaf HR reaction		
PMs PR10-1	6/7	6/7	0/3		
pG3-3 (35S)	3/3	3/3	2/2		

#### Legend to Table 2:

The results are shown as the number of plants responding positively to the GUS histochemical test as compared with the number of plants which were analysed.

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# B) Quantitative expression of the gus intron gene under the control of the different promoters

The method is based on an enzyme test.

The method employs:

- 15 a) a crude extract of the enzyme encoded by the gus gene, with the enzyme being obtained from transformed tobacco plants, and
  - b) a substrate, i.e. p-nitrophenyl-glucuronide. The rate at which the substrate is hydrolysed is monitored in a spectrophotometer and is related to the total quantity of protein in the extract. On the other hand, since it is not very sensitive, the method requires the presence of a strong promoter upstream of the gus gene.

As a consequence, it was not used for assays 25 which required 12 hours or more of incubation with the substrate which was used for the histochemical test (X gluc: 5-bromo-4-chloro-3-indolyl- $\beta$ -glucuronide).

Under these experimental conditions, the 35S promoter, located in plasmid pG3-3, gave a rate of substrate hydrolysis (expressed in arbitrary units) which was 5 times greater than that achieved using the

PMs PR10-1 promoter, which was located in plasmid pPR97. On the other hand, the PMs PR10-1 promoter gave a stronger expression of the gus gene than did the 35S promoter when the latter was located in plasmid p35S-gus intron. In fact, no substrate hydrolysis was detected in this latter case.

Similarly, it was not possible to obtain spectrophotometrically detectable values when the other constructs were used.

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#### Example 5

# Isolation of the DNA corresponding to a stilbene synthase gene and expression of stilbene synthase

Two methods were used for obtaining a gene 15 encoding grapevine stilbene synthase. On the one hand, data in the literature (WIESE et al., 1994) allowed the sequence of a gene to be obtained. On the other hand, a genomic insert of approximately 13 kb was supplied by BAYER AG (Agrochemical Division Research/Biotechnology-20 Pflanzenschutzzentrum, MONHEIM, D-51368 LEVERKUSSEN). It is specified that the company BAYER has deposited with the Deutsche Sammlung von Mikroorganismen (DSM), Germany, strains of E. coli containing plasmids bearing grapevine stilbene synthase genes (cf. EP-464 461): E. coli strain Fier 1 pVst 1 (DSM 6000, deposited on 18 25 June 1990), E. coli strain Fier 2 pVst 2 (DSM 6003), deposited on 18 June 1990) and E. coli strain Fier pVst 1 2 to 3 (DSM 6346, deposited on 11 February 1991). BAYER has also deposited the E. coli strain Nurdug 2010 deposited on 17 4243, September 1997) which 30 (DSM contains the plasmid pGS 828.1 which bears a groundnut stilbene synthase gene (cf. EP-309 862). The insert used carrying out the work reported below corresponds to a complex genomic clone which comprises two complete functional stilbene synthase gene sequences 35 (vst1 and vst2 genes) and an incomplete vst3 sequence. Subsequently, the sequence of the vst1 gene was chosen incorporation into the constructs produced. corresponds to a 4.9 kb genomic fragment (functional

sequence including the promoter) which does not possess any restriction sites which are suitable for cloning it directly into the plasmids which are usually used as transformation vectors. Additional sites were therefore 5 added to it by carrying out an intermediate cloning in a plasmid termed pCDNA II.

## A) Addition of supplementary sites by intermediate cloning in a plasmid pCDNA II

The abovementioned genomic fragment of the vst1 gene was isolated from the original plasmid in which it had been cloned in the form of an EcoRI/PstI fragment (2.1 kb) and cloned again into a plasmid pUC19. Having been cloned, the vst1 fragment was incorporated into the 15 same sites (EcoRI/PstI) of plasmid pCDNA II in order to change the restriction sites, delete the terminator of the gene and enable a BamHI/BamHI (1.8 kb) insert to be isolated. It was this fragment, corresponding to the open reading frame of the gene, which was used for 20 making the constructs with the different promoters including those isolated from the lucerne library (cf. Figure 4). This insert was then cloned into pBIN 19 after using Southern blotting to verify the sizes of the different fragments which were obtained 25 after digestion with appropriate restriction enzymes.

## B) Study of the expression of the vst1 gene

In order to verify the expression of the genes encoding stilbene synthase in the grapevine plants, a 30 probe (1.8 kb) encompassing the vst1 gene was prepared from plasmid pCDNA II, which had been multiplied in the E. Coli HB 101. The probe bacterium biotinylated by random priming, using the Polar Plex kit (Plex chemiluminescent kits, Millipore) in order to 35 enable genes or transcripts encoding a stilbene synthase to be detected by chemiluminescence on Southern and Northern blots carried out using nucleic acids which were extracted from control or transformed grapevine plants.

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1) Use of the vst1 probe for analysing genomic DNA extracted from grapevine 41B (V. vinifera Chasselas x V. berlandieri hybrid; stock-vine)

Southern blot analyses were carried out after extracting genomic DNA and then digesting it with EcoRI. Use of the vst1 probe enabled a large number of bands (approximately 15) to be obtained. These bands in fact correspond to fragments which contain sequences encoding 10 a stilbene synthase and which constitute a multigenic family (from 6 to 8 genes according to WIESE et al., 1994). Of these, vst1, vst2 and vst3 exhibit strong homology with each other. Furthermore, other genes can be recognized by this probe, in particular those 15 corresponding to the chalcone synthetase multigenic family. This is because the two enzymes have the same structure and are of the same size (dimers of subunits of from 41 to 44 kb in size). They also use the same substrate and their amino acid sequences exhibit a high 20 degree of homology, at least with regard to the active site.

The DNA of two plasmids was extracted for the purpose of verifying whether such a cross-hybridization was possible. One of the plasmids, pCDNA II, contained 25 the vst1 gene while the other, pPCV 002, contained a gene, available the chalcone synthase laboratory. A biotinylated probe corresponding to the Rosier chalcone synthase gene was also prepared. Southern blots obtained by carrying out cross-30 hybridization showed that the vst1 probe actually recognized the Rosier chalcone synthase fragment, and vice versa. The emitted signals are then weaker in this case of cross-hybridization.

2) Assay of resveratrol using grapevine plant 35 leaves

The fresh plant material, leaves or stalks, is reduced to powder in a mortar containing liquid nitrogen, and the apolar compounds are extracted with methanol (1 ml per 100 mg of fresh material : f.m.).

After centrifugation to remove debris, the methanol extract is filtered through a 0.45  $\mu m$  filter and then evaporated to dryness under nitrogen. residue is taken up in pure methanol (100  $\mu$ 1/100 mg In order to remove pigments (in particular chlorophylls), the sample is then passed over a C18 which has been (Sep-pack WATERS), qualitative The equilibrated with methanol. quantitative analysis of the extracts is carried out by 10 H.P.L.C. (High Pressure Liquid Chromatography) on a WATERS H.P.L.C. (Model 600 E) coupled to a diode-array 990. WATERS). The chromatography (Model support is composed of a reverse phase C18 column (C18 ultra base, 205  $\times$  4.6 mm, 5  $\mu$ m; Shandon). The analysis 15 of the extract compounds is carried out under isocratic conditions, the mobile phase consisting of a 35/65, V/V, acetonitrile/water mixture with a flow rate of  $1 \text{ ml} \times \text{min}^{-1}$ .

An absorption spectrum is performed every two seconds between 200 and 400 nm, and the resveratrol is detected at its adsorption maximum at 305 nm.

The quantification of the resveratrol is carried out by external calibration using a standard straight line obtained by chromatography of solutions at 5, 10, 20, 50 and 100 µg · ml<sup>-1</sup>, carried out with commercial resveratrol (Sigma). The resveratrol concentration, which is measured from the area under the peak corresponding to the molecule, is related to the unit mass of f.m. or of d.m. (dry matter) or chlorophyll mass of the sample assayed.

#### Example 6

Nucleic acid constructs which link the gene encoding a grapevine stilbene synthase to different promoters

35 1) Constructs using constitutive promoters

Two related promoters were used for making the genetic constructs permitting constitutive expression.

In the first construct, the DNA of grapevine stilbene synthase (vst1) was placed under the control of

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a regulatory sequence which consisted of a cassette containing two CaMV 35S promoters arranged in series (in the same orientation). A 35S polyadenylation sequence was also added to the end of the sequence encoding the vst1 gene. The chimeric construct which was made in this way can therefore be summarized as follows:

(CaMV) p35S - (CaMV) p35S - vst1 - (CaMV) 35S poly A

In the second construct, the *vst1* coding sequence was placed under the control of four enhancer sequences, which sequences were isolated from the CaMV 35S promoter and arranged in series upstream of the CaMV 35S promoter and the native enhancer sequence of the promoter of the grapevine gene (*vst1*). The two chimeric sequences which were produced in this way were first of all inserted into the plasmid PMP 90RK and the plasmids were then subsequently incorporated into the Agrobacterium strain GV3101.

These two regulatory sequences are regarded as being "strong" constitutive promoters.

2) Homologous construct using the 13 kb insert (vst genes under the control of their native promoters)

The grapevine genomic DNA fragment of approxiwas described above. 13 kb in size mately encompasses, in particular, two functional genes (vst1 and vst2) encoding the stilbene synthase enzymes and one incomplete (non-functional) gene (vst3). This grapevine sequence was cointegrated into a plasmid pGV3850, which was then introduced into an Agrobacterium strain. The sequence therefore corresponds to the open reading frames of vst (grapevine stilbene synthase) genes under the control of their native promoters.

Several series of 41B plants, which were transformed with the plasmid containing the 13 kb fragment, were obtained and were studied and analysed by Southern blotting using three different probes (1.8 kb probe from the nptII gene; 2.4 kb probe from the gene for resistance to ampicillin and 1 kb probe from the lefthand border of plasmid pBIN 19, comprising the final

integration sequence of the TDNA). The majority of the plants which were used reacted to one or other of these probes. These clones were numbered according to a code: 55 for the construct and 2, 3, 5, 6, 7 and 9 for the different transformants which were obtained.

3) Constructs using the inducible PMs PR-10-1 promoter

The construct containing the PMs PR-10-1
promoter, which was isolated from lucerne genomic PR
10 clones, was made (cf. Figure 5).

# Construction of the plasmid pBin 19 - PMs PR-10-1 - vst1-35S terminator gene

Since the PMs PR10-1 promoter which is isolated each contain and the *vst1* gene lucerne 15 from translation-initiating ATG codon, an adaptor was made in order to clone the vst1 gene into the construct without an additional ATG. This adaptor was synthesized in the form of two oligonucleotides of 11 bp in each case, one 20 of which was Bam HI-compatible while the other was Mun I-compatible. The adaptor was then incorporated into the Mun I site of the vst1 gene which was cloned into the plasmid pUC19. The insert was then recovered by digesting with Bam HI, after which it was cloned into 25 pBIN 19 between the PMs PR10-1 promoter and the 35S terminator. The insert of the vst1 gene, together with its adaptor, is therefore located between the PMs PR10-1 promoter and the 35S terminator. Under these conditions, the ATG of the vst1 gene was removed and 3 additional codons were included in the vst1 coding frame upstream of the gene. It was checked by sequencing that the open reading frame of the gere was still in phase with the remainder of the construct.

After having transformed 41B plants with the insert containing, inter alia, the chimeric sequence:

PMs PR10-1-promoter-vst1 gene 35S terminator, the transformants were analysed by Southern blotting using the nptII probe which has already been described. Nevertheless, it is not fully possible to demonstrate

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using this method and, in complete integration since, in the Agrobacterium this probe, particular, system, it is accepted that insertion into the genome of the plant begins at the right-hand border and terminates 5 at the left-hand border. Since, in the construct which is used, the nptII gene is located close to the rightpossible for therefore border, it is integration to be partial, with the nptII gene being inserted but with there being a subsequent block in 10 integration before the left-hand border is reached. The transformants were coded 145 and allocated the numbers 2, 5 and 6 for those transformants whose results are presented below.

# 15 Example 7 Genetic transformation of the grapevine and analysis of the efficiency of the promoters being studied

As described above (Example 6), four main constructs were produced for transforming the 41B stock-20 vine ( $V.vinifera \times V.$  berlandieri) laboratory model system. The reason for using the latter system is that it has the advantage of giving good results in the embryogenic cell suspensions with transformation of transformants 50 are agrobacteria. Approximately 25 obtained on average in experiments using from 0.1 to 1  $\mu$ l P.C.V. (packed cell volume) of embryogenic cells. Furthermore, selection, development of the transformed embryos and regeneration into plants are all rapid. Plantlets having from 6 to 8 well-developed leaves can 30 be obtained in vitro in two months of culture.

# A) Genetic transformation of 41B with vectors containing the *vst1* gene under the control of constitutive promoters

Two constructs were tested, the one containing the 35S double promoter arranged in series upstream of the *vst1* gene and the second consisting of a regulatory sequence composed of 4 CaMV 35S enhancer sequences arranged in series upstream of the CaMV 35S promoter,

with the whole being located upstream of the vst1 gene possessing its native enhancer sequence. Four trial series were performed (two for each of the constructs) with the aim of transforming the embryogenic grapevine 5 cells. None enabled plants or even embryos to be regenerated. In every case, rapid necrosis of the embryogenic cell suspensions was obtained after the 48 hours of coculture with the agrobacteria. These constructs cannot obtain transformed plants which to are be used constitutively expressing the vst1 under the gene control of these "strong" promoters. One hypothesis may be put forward; it is possible that expression of this gene blocks the regeneration into embryos due to the rapid necrosis of the potentially embryogenic cells in 15 response to the production of stilbenic phytoalexins.

These negative results, which were obtained with the constructs containing constitutive promoters of 35S type, demonstrate the importance of controlling overexpression of the *vst.*1 gene by means of a homologous or heterologous promoter which can be induced, in particular, by a pathogen.

These present results are similar to those which were published by FISHER and HAIN in 1994 and which underline the fact that they did not succeed in groundnut stilbene expressing the 25 constitutively synthase gene at an elevated level in tobacco when using the CaMV 35S promoter. According to these authors, when such a construction is used, expression of the gene would be regulated negatively when the plant is attacked 30 by a pathogen. This regulation, according to their hypothesis, could result from the deployment, by the plant, of defence mechanisms which are normally induced (synthesis of PR proteins, the pathogens particular) and which would exert an inhibition on the 35 viral promoters.

- B) Genetic transformations of 41B with vectors containing the *vst1* gene under the control of promoters which can be induced by abiotic and/or biotic stresses
- 5 1) Study of the expression of the *vst* genes, and of their kinetics of induction with UV light, in excised leaves which are isolated in the surviving state, and in whole-plant control 41B grapevine vitroplants or 41B grapevine vitroplants or which are transformed with the 10 13 kb insert

It has been demonstrated that the synthesis of resveratrol (main grapevine phytoalexin), which is a product of the reaction catalysed by an enzyme stilbene synthase can be induced by ultraviolet (UV) light, that is under conditions of abiotic stress (LANGCAKE et al., 1977; SBAGHI et al., 1993). Under normal conditions, grapevine synthesizes little or no resveratrol; by contrast, following induction with U.V. light (10-minute exposure to UV light at 254 nm, for a lamp dissipating 600 μW.cm<sup>-2</sup>), followed by a period of 20 hours in the dark, the phytoalexin is synthesized in the excised leaves.

#### a) Method used

- 25 Irradiation of the leaves:
  - leaves of vitroplants: at 254 nm for a lamp dissipating 600  $\mu W.\, cm^{-2}$  vitroplants for 13 minutes,
  - leaves isolated from vitroplants or at 254 nm for a lamp dissipating 600  $\mu W.\, \text{cm}^{-2}$  for 8 minutes,
- 30 extraction and analysis of the mRNA from the plant material and estimate of the resveratrol by fluorescence after excitation at 365 rm at a given time after induction.

Each sample consists of 3 leaves which have been isolated from the same plant and which have been separately induced by UV light but then recombined for the extraction and assay. The test is performed on excised leaves, which have been isolated from vitroplants or on

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vitroplants which are being cultured on agar medium and which possess from 6 to 7 well-developed leaves. The three oldest leaves on each plantlet are removed and used for the test. The upper surface of the leaves is exposed to the UV light. Following analysis, the quantities of resveratrol obtained are expressed in µg of product either per gram of fresh weight of analysed leaves or per gram of dry matter (estimated on the pellet after centrifuging and after extraction with methanol) or in mg of resveratrol per g of chlorophyll.

The tables given below show the values which were obtained for the 41B and 55-X clones, which were transformed with the 13 kb genomic clone construct in which two genes, i.e. vst1 and vst2, both of which genes are under the control of their native promoters, are present in the sequence used as insert.

# b) Study of abiotic (UV light) stress on excised leaves (experiment No. 1)

20 The results, corresponding to construct 55 (13 kb insert) and to clones 2 (55-2) and 3 (55-3), are presented in Tables 3 and 4 below (assay of resveratrol) and depicted in Figures 6 and 7 (analysis of the transcripts). The study of the kinetics of the induction by UV light of the expression of the genes encoding stilbene synthase was performed after a period which separated the induction with UV light from the analyses and which was either fixed at 17 hours (cf. Table 3 and Figure 6) or was a variable period of 0, 8, 17, 24 or 32 hours after induction (cf. Table 4 and Figures 7 and 8).

Table 3: Quantities of resveratrol which are detected in uninduced control and transformed leaves or in control and transformed leaves at 17 hours after induction with 35 UV light (expressed in  $\mu g.g^{-1}$  of fresh material)

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	Untransformed Clone 55-2 controls		55-2	Clone	55-3
Uninduced	Induced	Uninduced	Induced	Uninduced	Induced
0	12	0	11	0	13

Legend to Table 3: The leaves were excised from 41B vitroplants prior to induction with UV light. Clones 55-2 and 55-3 correspond to transformed plants which have integrated an insert of 13 kb which contains genes encoding stilbene synthase.

The fluorescence, which is observed in the blue/violet band at approximately 450 nm, is very strong in the veins of the excised leaves and is distributed uniformly over the entire surface of the leaf. The control leaves, which have not been induced with UV, do not exhibit any fluorescence. These results demonstrate specific expression of the genes encoding stilbene synthase. Analysis of the transcripts by means of Northern blotting, which was carried out using the vst1 probe, demonstrates the presence of a fragment of approximately 1.8 kb in size whose emitted signal is very intense in the case of the leaves induced with UV light but which is absent or very weak in the case of the uninduced plants (cf. Figure 6).

Table 4: Kinetics of the development of resveratrol concentrations in 41B vitroplant leaves which have been induced with UV light, removed from the plants being cultured on agar medium, and then isolated and analysed for their resveratrol content at various periods after induction.

Time after induction (h)	Quantity of resveratrol (in µg.g <sup>-1</sup> of fresh material)		
	Control	PCT 55-2	PCT 55-3
NI	0	0	0
0	0.6	0	0
8	30.8	12.1	9
17	56.8	71.7	47
24	83.2	81.4	35.2
32	15.5	7.7	151.8

Legend to Table 4: The results are expressed in μg.g<sup>-1</sup> of fresh material. Two plants, transformed with PCT 55-2 and PCT 55-3 respectively, were analysed in comparison with the control.

NI: Not induced with UV light.

Under these conditions, after stressing with UV light, the quantity of resveratrol found in the control leaves is at a maximum at 24 hours after induction (of the order of 80  $\mu g.g^{-1}$  of f.m. for 41B). However, there are significant variations depending on the date of the analysis in the cycle of subculturing the vitroplants: micropropagation cycle.

The results obtained after Northern blot analysis using the vst1 probe are depicted in Figure 7. At least two types of transcripts, of very similar size (approximately 1.8 kb), were detected. Although cross-hybridization with a chalcone synthetase transcript cannot be excluded, the different messenger RNAs which were recognized probably correspond to the expression of different stilbene synthase genes. Thus, it has been shown in the grapevine (WIESE et al., 1994; KINDL, personal communication) that differences exist in the kinetics of induction of the different genes of the

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multigenic family encoding stilbene synthases.

Northern blot analysis demonstrated that, in the leaves which were excised from grapevine 41B and then subjected to the abiotic stress which exposure to UV 1 light represents, the stilbene synthase transcripts exhibit an expression maximum at approximately 17 hours to 32 hours after induction. These results are comparable with those obtained with grapevine cell cultures, which also demonstrated the same pattern of expression but with the presence of two maxima (WIESE et al., 1994).

c) Study of abiotic stress (UV light) in the isolated surviving leaf (experiment No. 2), induction in vitroplants before isolation of the leaves

Table 5 below presents the results which were obtained for a second series of transformants which had integrated the 13 kb insert. The study of the kinetics of the induction, with UV light, of the expression of the genes encoding stilbene synthase was performed after a variable period, separating the induction with UV light from the analyses, of 20, 40 or 60 hours.

Table 5: Concentration of resveratrol in the isolated 25 leaves of grapevine vitroplants after induction with ultraviolet light and extraction at different survival times.

Survival time of the leaves isolated from vitroplants after induction with UV light (8 min at 254 nm) and before extraction of the resveratrol

Clone studied	20 hours	40 hours	60 hours
41B - control not induced	0	0	0
41B - control induced	281	165	6
Clone 55-2 induced	135	918	58
Clone 55-3	44	931	301
Clone 55-5 induced	392	83	657
Clone 55-6 induced	339	235	43
Clone 55-7	263	411	148
Clone 55-9 induced	386	823	54

### Legend to Table 5:

The concentrations of resveratrol are expressed in  $\mu g.g^{-1}$  5 of dry matter.

41B stock-vine hybrid V. vinifera Chasselas  $\times$  V. Berlandierri.

The results presented in Table 5 enable two groups of plants to be distinguished:

10 - The first corresponds to the control and to two of the transformants, i.e. 55-6 and 55-7. In general, they exhibit a maximum resveratrol concentration of between 280 and 410  $\mu g.g^{-1}$  of dry material, with this maximum

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generally being at 20 hours after induction, except for clone 55-7, when it is at 40 hours.

The second group exhibits maxima which are higher, that is almost double those of the first group (from 820 to 930 μg.g<sup>-1</sup> of dry material). This group consists solely of transformants (55-2, 55-3 and 55-9). The maximum is expressed at 40 hours after the induction with UV light; on the other hand, at 20 hours after induction, these plants frequently have concentrations which are much lower than those of the first group. This is the case, for example, for clones 55-2 and 55-3 (135 and 44 μg.g<sup>-1</sup> of dry material, respectively). However, one of the clones, i.e. 55-9, is of interest since it exhibits resveratrol concentrations which are greater than those of the control in all cases (386, 823 and 54 μg.g<sup>-1</sup> of dry material at the times of 20, 40 and 60 hours after induction).

In this surviving leaf system, an uninduced control never exhibits resveratrol and, in almost all cases (apart from clone 55-5, which behaves in a distinctive manner), the resveratrol concentration falls drastically at 60 hours after induction.

# d) Study of abiotic stress (UV light) which is carried out on vitroplants which are being cultured on agar medium

This study, which is carried out on a plant which is growing on an agar medium, makes it possible to analyse the production of resveratrol when other defence mechanisms of the plant may be being expressed, at least those mechanisms which are capable of being expressed under the in-vitro culture conditions. Furthermore, the study makes it possible to monitor the synthesis of resveratrol over a longer period (in the preceding studies, the isolated leaf necroses beyond 72 hours).

Four transformed 41B vitroplant clones in culture (55-2, 3, 5 and 6) were treated with UV light under the same conditions as before (8 min at 254 nm), with the leaves only being removed from the plant at

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20 hours after induction. The results which were obtained were compared with the untransformed control as well as with a clone of *Vitis rupestris* and with 3 clones of *Vitis vinifera* variety which are known in the field for having a relatively high susceptibility to attacks by *Botrytis* on their berries.

The literature (SBAGHI et al., 1993) in fact demonstrates that a correlation exists between the susceptibility of the berries to *Botrytis*, as assessed in the vineyard, and the content of resveratrol in the leaves of vitroplants which had been induced with UV light. The results are presented in Table 6 below.

Table 6: Results of HPLC assays of resveratrol which were performed at 20 h after inducing several different varieties of grapevine with UV light of 254 mm for 8 min.

Variety tested	(resveratrol) μg.g <sup>-1</sup> of dry weight
Rupestris 215	351
Stock-vine 41B	237
Ugni-blanc 479	209
Pinot noir 386	86
Folle blanche 280	37
Pct 55-2	361
Pct 55-3	235
Pct 55-5	115
Pct 55-6	539

## 20 Legend to Table 6:

The induction with UV light is performed on vitroplants which are being cultured on agar medium. The leaves are removed and extracted for analysis at 20 hours after the treatment.

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PCT 55-2, 3, 5 and 6 - transformants which have integrated the 13 kb insert into their genome. The results show the average of 3 repetitions.

These results provide a good indication that a 5 correlation exists among the varieties and grapevine plants between susceptibility to Botrytis and content of resveratrol in the leaves at 20 hours after induction. Two groups can be identified in the untransformed varieties and grapevine plants. The first, which is 10 formed by V. ruspestris, V. vinifera x V. berlandieri (41B) and Ugni-blanc, corresponds to those which are relatively tolerant to the fungus. The consisting of the Pinot noir and the Folle blanche, represents those which are regarded as being moderately 15 tolerant to very susceptible (Folle blanche example).

As far as the transformants are concerned, they have, on average, resveratrol contents at 20 hours after induction which are greater than or equal to the untransformed 41B control (539, 362 and 236 µg.g<sup>-1</sup> of dry material for clones 55-6, 55-2 and 55-3, respectively, and 237 for the control).

By contrast, the concentration obtained in the case of the transformed clone 55-5 is half that of the control. If these values, expressed in  $\mu g.g^{-1}$  of dry weight, are compared with those of the isolated leaves (Table 5), it is seen that they are similar in the case of the control but that, on the other hand, variations exist in the case of the transformants which are analysed at 20 hours after induction. These variations are at times very substantial (135 for induction in isolated leaves and 362 for induction in vitroplants in the case of clone 55-2; 44 against 236 in the case of clone 55-5 and 339 against 540 in the case of 55-6). In addition, a great variability exists among the various repetitions.

2) Comparative study of the expression of the *vst* gene and of its kinetics of induction by biotic stress in 41B

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grapevine vitroplants which have been transformed with the 13 kb insert and with the construct comprising only the *vst1* gene under the control of the PMs PR10-1.

The results above (chapter 1), which were obtained in vitroplants which had integrated, by genetic transformation, additional copies of stilbene synthase genes (in the form of a 13 kb insert comprising two functional vst1 and vst2 sequences), show that an overproduction of resveratrol, which is produced from the reaction catalysed by the stilbene synthase enzyme, is possible in some transformants when these genes are under the control of their own promoters, and in response to an abiotic stress such as U.V. irradiation.

The production of resveratrol was then verified with respect to these results, in two types of transformant, the first representing a 41B clone which had integrated the 13 kb insert (vst1 and vst2 stilbene synthase genes under the control of their own promoters), the second representing several clones which had incorporated only the vst1 gene under the control of the lucerne defence gene regulatory sequence PMs-PR10-1. This comparison was carried out after induction by a biotic stress caused by Botrytis cinerea.

### 25 a) Methods used

# α) Demonstration of the fungitoxic effect of the resveratrol molecule on *Botrytis cinerea*

The data in the literature with regard to the fungitoxic nature of the molecule are contradictory. According to DAÏ (1994), resveratrol does indeed exhibit an inhibitory effect on the development of the zoospores of Plasmopora viticola (agent of mildew); by contrast, PONT and PEZET (1990) maintain that it does not block germination of Botrytis cinerea conidia.

A study was carried out of the action of the molecule on the growth of mycelial hyphae of Botrytis cinera in culture on a malt/agar medium containing a resveratrol dilution range of from  $10^{-1}$  M to  $3.7.10^{-3}$  M,

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with the hyphae being incubated at ambient temperature for 7 days. The results demonstrated that the molecule had an inhibitory effect (IC<sub>50</sub> = 500  $\mu$ mol.1<sup>-1</sup>), with an exponential decrease in the mean growth diameter of the 5 fungus for concentrations greater than 125  $\mu$ mol.1<sup>-1</sup>. On the other hand, concentrations of 37  $\mu$ mol.1<sup>-1</sup> only have a very slight inhibitory activity on the growth of the mycelium (cf. Figure 9). However, this inhibition increases progressively after 7 days of culture under 10 these conditions, which are otherwise very favourable to the growth of the fungus. After 20 days, the fungus finally infects the whole of the culture surface.

# $\beta$ ) Screening test for the tolerance of grapevine vitroplants to Botrytis cinerea

The implementation of the test consisted in inoculating the leaves of plantlets, which were being cultured in vitro on micropropagation medium, by depositing 20  $\mu$ l of a suspension of conidia, containing  $1.10^{-4}$ conidia/ml (200 conidia per deposition) in malt/glucose 20 medium on their upper surface. The plantlets, different leaves of which had thus been inoculated, were then cultured in a climate chamber (photoperiod: 16 h day, 8 h night; temperature: 24°C; humidity: 70%). Two days after the inoculation, the leaves of row 4, i.e. 25 the youngest leaves, were inspected (the necroses and macerations which were present were counted using a camera which was linked to a television monitor) and order extracted with methanol to assav in resveratrol which had been synthesized in response to 30 the Botrytis attack. At 5 days, the number 2 leaves were removed for observation by fluorescence microscopy. Macroscopic observation (necroses and macerations in the leaves) and counting of the leaves exhibiting fruiting bodies of the fungus (conidiophores) were also performed 35 on the number 3 leaves. Finally, at 9 days, leaves which were interacting with the fungus, and which had been removed from three different plants, were extracted with methanol in order to assay the resveratrol.

γ) <u>Induction of biotic stress by depositing a suspension containing Botrytis cinerea spores on vitroplant leaves</u>
-test of tolerance to <u>Botrytis</u>

This direct confrontation test was performed using the technique which has already been described (cf. the preceding paragraph). The same applies to the observations which were made. Four vitroplants were used for each variety or transformed clone and, in the case of each of these vitroplants, three leaves which had developed in rows 2, 3 and 4 were inoculated with the conidial suspension (200 conidia in 20  $\mu$ l of malt/glucose medium). 12 inoculations were therefore performed for each variety or clone and the following analyses and observations were made:

- 15  $\rightarrow$  Two days after inoculation:
- inspection of the leaves in row 4 for foliar symptoms (fungal maceration zone or necrotic spots, which consist of small blackish brown regions which are located around the fungal spores, or without visible symptoms) and,
  - assay of resveratrol in the same leaves.
  - → Five days after inoculation:
- inspection of the leaves in row 3 for foliar symptoms with counting of those leaves which carry
   conidiophores (fruiting bodies of the fungus) and
  - fluorescence microscopic inspection of the leaves in row 2 in order to locate the regions of resveratrol synthesis.
  - → Nine days after inoculation:
- assay of resverative in three leaves which are derived from three different plants and which are interacting with the parasite.

For each experimental series, three Vitis vinifera varieties having different susceptibilities to Botrytis attack on their leaves were studied: Folle blanche (susceptible), Pinot noir (moderately tolerant) and Ugni blanc (tolerant), together with the stock-vine 41B (tolerant) as well as four of its transformants: one having inserted supernumerary copies of the genes

encoding a stilbene synthase (13 kb insert), i.e. clone 55-3, and the three others representing transformants harbouring the PMs PR10-1 promoter-vst1 gene construct, that is clones 145-2, 145-5 and 145-6.

### 5 b) Results obtained

The results for the foliar symptoms observed at 2 or 5 days after the inoculation are presented in Tables 7 and 8 and the results for the assays of resveratrol, related either to dry weight or to chlorophyll content, are presented in Tables 9 and 10.

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<u>Table 7</u>: Macroscopic observations of vitroplant leaf/Botrytis cinerea interactions at 2 days

Variety tested	Number of No. 4 leaves observed in 4 different plants in which the following symptoms appear		
	Regions of	Necrotic spots	No visible symptom
Folle blanche 280	4	1	0
Pinot noir 386	3	4	0
Ugni-blanc 479	1	2	1
41B	0	4	0
Pct 55-3	2	3	0
Pct 145-2	0	3	1
Pct 145-5	0	1	3
Pct 145-6	1	1	2

## 5 Legend to Table 7:

- Regions of maceration: broad and diffuse region in which *Botrytis* is rapidly destroying the plant cells. These regions have a light brownish beige colour.
- Necrotic spots: very small regions centred around the fungus. These spots have a blackish brown colour.

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<u>Table 8</u>: Macroscopic observations on the vitroplant leaf/Botrytis cinerea interactions at 5 days

Variety tested	Number of No. 3 leaves observed in 4 different plants in which the following symptoms appear		
	Regions cf	Necrotic spots	No visible symptom
Folle blanche 280	4	0	4
Pinot noir 386	3	1	2.5
Ugni-blanc 479	2	1	0.5
41B	1.5	2	0.5
Pct 55-3	2	2	1
Pct 145-2	1.5	2	1
Pct 145-5	0.5	4	0
Pct 145-6	3	2	1

## Legend to Table 8:

- Regions of maceration: broad and diffuse region in which *Botrytis* is rapidly destroying the plant cells. These regions have a light brownish beige colour.
- Necrotic spots: very small regions centred around the fungus. These spots have a blackish brown colour.

<u>Table 9</u>: Results of the HPLC assays of resveratrol which were carried out on different grapevine varieties which had been interacting with *Botrytis cinerea* for 2 days

Variety tested	Resverat.  mg.g <sup>-1</sup> of chloroph.	Resverat. μg.g <sup>-1</sup> of dry weight
Folle B280	5.1	101
Pinot N386	4.3	102
Ugni B479	3.9	86
Stock-g41B	5.7	112
Pct 55-3	4.6	118
Pct 145-2	6.9	170
Pct 145-5	2.2	83
Pct 145-6	4.3	107

## Legend to Table 9:

- Resverat.: resveratrol; chloroph.: chlorophyll
- 5 Folle B 280: Folle blanche 280; Pinot N 386: Pinot noir 386; Ugni B 479: Ugni-blanc 479; Stock-g41 B: Stock-vine 41B

Table 10: Results of HPLC assays of resveratrol which were carried out on different grapevine varieties which had been interacting with *Botrytis cinerea* for 9 days

Variety tested	Resveratrol mg.g <sup>-1</sup>	Resveratrol µg.g <sup>-1</sup> of dry weight
Folle blanche 280	4.0	38
Ugni-blanc 479	31.7	145
Stock-vine 41B	11.9	59
Pct 145-5	602.1	2558

....

...

→ Two days after inoculation:

The observation related to the youngest leaves 4). Regions of maceration (colonization by the fungus) were observed in almost all cases, apart from and 145-5. The 145-2 and clones grapevine varieties exhibited these regions to a greater extent than did the tolerant varieties: Folle blanche and Pinot noir exhibited 4 and 3 regions, respectively, as against only 1 in the case of the Ugni blanc variety. 10 Only the 145 transformants (PMs PR10-1 promoter-vst1 gene) and the Ugni blanc gave leaves without visible symptoms. Necrotic regions, which are a plant defence reaction, appeared mainly in 41B and its transformants and, in the case of the grapevine varieties, in Pinot 15 Noir and Ugni blanc.

If these observations are compared with the resveratrol assays (Table 9), it is not possible to establish any correlation since, when related to dry weight, the contents of resveratrol in these leaves are comparable with a value of approximately 100 µg.g<sup>-1</sup> of dry material. In all the vitroplants examined, the contents settle at values of between 4 and 5 mg.g<sup>-1</sup> of chlorophyll.

It is only the stock-vine 41B and, in 5 particular, transformant 145-2 which have higher values. Clone 145-5 has a lower value (approximately half the size),

A comparison can also be made with the resveratrol contents which were previously obtained at 20 hours
30 after inducing the vitroplants with UV light (Table 6).
Although the period of sampling is not comparable
(20 hours in the case of the abiotic stress as compared
with 48 hours in the case of the biotic stress, although
the time necessary for the germination of the spores has
35 to be taken into account), lower contents of resveratrol
are generally observed after a biotic stress (half or
one third, in the case of the Ugni blanc, of the
amount), apart from the Pinot noir, where the content is
comparable, and the Folle blanche, where the content is

approximately three times greater.

In all the samples analysed, it may be emphasized that there was a marked scattering of the values obtained in the different repeat assays which were performed. This variability between different leaves of one and the same plant, which was previously observed with the samples which had been subjected to the UV light inductions, could, in the latter case too, be due to several different factors. The most plausible hypotheses which may be mentioned are:

A wide variability between vitroplants of one and the same clone in their reaction in the face of fungal attack. The variable foliar symptoms which were obtained following inoculation with the *Botrytis* spores appear to confirm this (variability in the infection, in the physiological state of each plant, etc.).

The assay, which is performed on the whole leaf, is not representative of the variations in the synthesis of resveratrol which exist in each cell which forms the 20 leaf. Thus, it is generally acknowledged that, in hypersensitivity reactions to a parasite, it is not all the cells of the lamina which synthesize defence molecules. It is only the cells which are located close to the regions of fungal attack which are induced to synthesize 25 phytoalexins. Therefore, the analyses which were performed in this test only represent values which correspond to a mean resveratrol level which is present in the lamina of leaves which are interacting with parasite. A sizeable dilution effect can therefore be 30 produced, in particular in the case of the resistant plants, between the concentrations which are present in the cells which are induced to synthesize phytoalexin and the value which is found when the whole leaf is analysed. This effect is doubtless more marked during the early stages of expression of the plant defence reactions.

→ Five days after the incculation:

The symptom observations show (photographic plates Nos. 1 and 2) (Figures 10 and 11) and Table 8)

that regions of maceration are formed, in relatively large numbers, in all the grapevine varieties, in the stock-vine and in the transformants which were studied. These maceration regions are often associated with the 5 presence of conidiophores (fruiting bodies fungus). Of the grapevine varieties and the control stock-vine 41B, the Folle blanche, which is the species which is most susceptible to Botrytis, shows attacks on all the leaves studied, and also conidiophores. If a severity of the symptoms 10 hierarchy in the established, the Pinot noir comes next, followed by the Ugni blanc and finally 41B. However, in the case of these two latter varieties, conidiophores only developed on one single half-leaf. As far as the transformant 15 clones are concerned, three clones gave reactions which were more or less similar: 55-3, 145-2 and 145-6. Only clone 145-5 exhibited good tolerance to the parasite since a region of maceration only developed on one halfleaf and no conidiophores were visible.

On the other hand, most of the leaves of this clone reacted to the attack by forming necrotic regions.

An example is also depicted in photographic plate 2 (Figure 11).

In order to verify whether these results corresponded satisfactorily to the marked differences in the 25 induction of resveratrol synthesis, and therefore to the expression of gene(s) encoding a stilbene synthase, leaves which were inoculated with the Botrytis spores were inspected by fluorescence microscopy (filter unit 30 A: excitation filter of from 340 to 380 nm; stop filter these conditions, chlorophyll 425 nm). Under fluoresces red while resveratrol fluoresces bluish white or a darker blue depending on its concentration. Two grapevine varieties, i.e. Folle blanche (susceptible) and Pinot noir (moderately tolerant), the stock-vine 41B (tolerant) and one of its transformants, i.e. 145-5 (PMs PR10-1 promoter- vst1 gene construct), were studied by this method. The photographs obtained from observations are presented in photographic plate 3

(Figure 12). Striking differences can be seen. In the case of the Folle blanche, the parasite mycelium (black in the photograph) has developed and has colonized the tissues. Practically no bluish cell is visible. In the 5 case of the Pinot noir variety, a bluish region can be observed, which region forms a barrier around and in the region of inoculation and incipient colonization by the fungus. The growth of the mycelium is retarded, if not blocked, by this barrier, even if a tissue maceration 10 process has already been instituted. A more intense blue coloration also exists in the veins. In the region studied in the case of the control stock-vine 41B, scattered cells, which are distributed throughout the lamina, display a light blue fluorescence with an 15 intensity which is greater in the veins. No fungal colonization process has been instituted, and scattered necrotic regions, which are limited to a few cells, can be seen. With regard to the 41B which is transformed with the PMs PR10-1 promoter-vst1 gene construct, the 20 results are spectacular in the case of clone 145-5. While incipient colonization of the tissues by the fungus has been instituted (black region), barrier, which is synthesizing resveratrol, has formed very rapidly around this region thereby preventing it 25 from being extended. Blue cells are still visible in the region of fungal maceration. Furthermore, many of the cells of the lamina of the leaf which are not in contact with the fungus have themselves synthesized resveratrol. The veins also display an intense blue coloration.

30 These observations demonstrate that a correlation therefore exists between the intensity of the foliar symptoms which developed after inoculating vitroplants with Botrytis spores and the content of stilbenic phytoalexin in the leaves. The most tolerant plants are those which display a resveratrol synthesis which is distributed over a large part of the lamina. This is the case with transformant 145-5, which harbours a gene encoding a stilbene synthase under the control of the PMs PR10-1 promoter, which promoter was isolated

from lucerne.

→ Nine days after inoculation:

The symptom observations demonstrated that the Folle blanche vitroplants were particularly susceptible to Botrytis attacks in this test as well. At nine days, they are all infested with the fungus and their chlorophyll is markedly degraded in most cases. With regard to the Ugni-blanc variety and the stock-vine 41B, it was not possible to observe any difference in expression of the foliar symptoms. Generally speaking, the symptom observation results which were obtained at 5 days are encountered once again while being a little more developed in the case of the regions of maceration. On the other hand, most of the leaves which exhibited conidiophores have necrosed.

4 varieties were analysed for their content of resveratrol: the Folle blanche (susceptible), the Ugniblanc (tolerant), the 4.B (tolerant) and 145-5 (41B transformed with the PMs PR10-1 promoter-vst1 gene).

20 Nine days after inoculation, 145-5 still did not exhibit any significant symptoms of Botrytis attack. The results of the resveratrol content analyses are presented in Table 10. When expressed in μg of resveratrol .g<sup>-1</sup> of dry weight or in mg.g<sup>-1</sup> of chlorophyll, the resveratrol concentrations can be used to rank the varieties in the following manner, proceeding from the lowest value to the highest: Folle blanche < 41 B < Ugni-blanc < the transformed 41B strain 145-5.

The concentration differences are very substan30 tial since the transformant 145-5 has a value which is
almost 43 times greater than that of the 41B control
(per g of dry weight) and 50 times greater if it is
expressed per g of chlorophyll. The assays therefore
provide good confirmation of the fluorescence microscopy
35 assessments that were made at five days (see photograph
plate 3, Figure 12).

With regard to the Ugni-blanc and the 41B, Table 10 shows that the former has approximately 3 times more resveratrol than the latter whichever units are

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used as insert.

selected. However, these two varieties react in an identical manner in terms of foliar symptoms. This similarity in tolerance can be explained by factors other than the synthesis of resveratrol. It must also be noted that, under the conditions of the test, the plant/Botrytis confrontation is particularly favourable to the latter. The environmental conditions which exist in a container in vitro ensure that, after approximately 20 days of culture, all the vitroplants, whatever their nature, are infected with the fungus.

c) <u>Conclusions on the genetic transformation of grapevine and analysis of the efficiency of the promoters studied</u>

It was not possible to regenerate plants containing the constructs which comprised the *vst1* gene under the control of the 35S promoter or its derivatives from the experiments on 41B (stock-vine hybrid *V. Vinifera x V. Berlandieri*) embryogenic cells which were transformed with *Agrobacterium tumefaciens* harbouring the different constructs. It was not therefore possible to obtain any strong constitutive activity over the whole of the plant.

By contrast, grapevine plants which had incorporated the PMs PR10-1-vst1 gene and 13kb insert constructs (vst1 and vst2 genes under the control of their native promoters) were obtained. It was possible to compare these genetically transformed plants with the control (41B untransformed) and multiply them by micropropagation.

Few differences were observed, as compared with the untransformed controls, in the concentrations of resveratrol which were obtained, under UV light stress (biotic stress), in the different transformants which were prepared with the 13 kb insert, in which two genes, i.e. vst1 and vst2, both of which are under the control of their native promoters, are present in the sequence

By contrast the results demonstrate that the chimeric PMsPR10-1-vst1 gene construct enables an

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approximately 50-fold overexpression of the phytoalexin resveratrol (product of reaction catalysed by a stilbene synthase) to be achieved, in the presence of a biotic stress caused by *Botrytis cinerea*, at 9 days after infection. These plants then demonstrate the best tolerance when they are compared either with the control or with the transformants which were obtained with the 13 kb insert.

Thus, transformation with the construct which links the inducible lucerne promoter PMs PR10-1 to the stilbene synthase genes enables the stilbene synthase genes to be overexpressed in grapevine plants in response to a stress such as pathogen attack.

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#### CLAIMS

- 1. Nucleic acid which comprises the sequence of the promoter for a lucerne PR protein linked to at least the sequence of a gene encoding a stilbene synthase.
- 5 2. Nucleic acid according to Claim 1, characterized in that the promoter for a lucerne PR protein is a promoter which can be induced in plants, in a tissue-specific manner or not, by a biotic or abiotic stress.
- 3. Nucleic acid according to one of Claims 1 and 2, characterized in that the sequence of the promoter for a lucerne PR protein is selected from the group comprising:
  - a) the IND S1 sequence,
- b) the sequence which is of a fragment of the IND 15 S1 sequence and which has a promoter sequence effect in plants, and
  - c) a sequence exhibiting at least 80% homology with the IND S1 sequence.
- 4. Nucleic acid according to Claim 3, characterized in that the sequence of the promoter for a lucerne PR protein exhibits at least 90% homology with the IND S1 sequence.
- 5. Nucleic acid according to Claim 3, characterized in that the sequence of the promoter for a lucerne PR 25 protein exhibits at least 95% homology with the IND S1 sequence.
  - 6. Nucleic acid according to one of Claims 1 to 5, characterized in that the sequence of the gene encoding a stilbene synthase is selected from the genes isolated from groundnut, orchid, grapevine and pine genomes.
  - 7. Nucleic acid according to Claim 6, characterized in that the sequence of the gene encoding a stilbene synthase is the sequence of a gene encoding a grapevine stilbene synthase.
- 35 8. Nucleic acid according to Claim 7, characterized in that the sequence of the gene encoding a grapevine stilbene synthase is a sequence selected from:
  - a) the vst1 gene

- b) the vst2 gene.
- 9. System for expressing a stilbene synthase gene in plants, characterized in that it comprises at least one nucleic acid according to one of Claims 1 to 8.
- 5 10. System for expressing a stilbene synthase gene in plants according to Claim 9, characterized in that the system is a vector.
  - 11. Expression vector according to Claim 10, characterized in that the vector is a plasmid.
- 10 12. Expression system according to one of Claims 9 to 10, characterized in that it can be transferred into Agrobacterium strains.
  - 13. Expression system according to one of Claims 9 to 12, characterized in that it can be induced in
- 15 plants by a biotic or abiotic stress.
  - 14. Expression system according to Claim 13, characterized in that the biotic stress is a parasite attack.
    - 15. Expression system according to Claim 14, characterized in that the parasite is a bacterium, a yeast, a
- fungus or a virus.

  16. Expression system according to Claim 15, characterized in that the parasite is *Botrytis cinerea* or
  - Plasmopora viticola.

    17. Expression system according to Claim 13,
- 25 characterized in that the abiotic stress is a mechanical wound.
  - 18. Expression system according to Claim 17, characterized in that the mechanical wound is caused by an insect.
- 30 19. Expression system according to Claim 17, characterized in that the mechanical wound is caused by a physical phenomenon such as wind or frost.
  - 20. Plant cell which is transformed with a system or a vector according to one of Claims 9 to 19.
- 35 21. Cell according to Claim 20, characterized in that it is a grapevine cell.
  - 22. Process for obtaining a cell according to one of Claims 20 and 21, characterized in that a plant cell is

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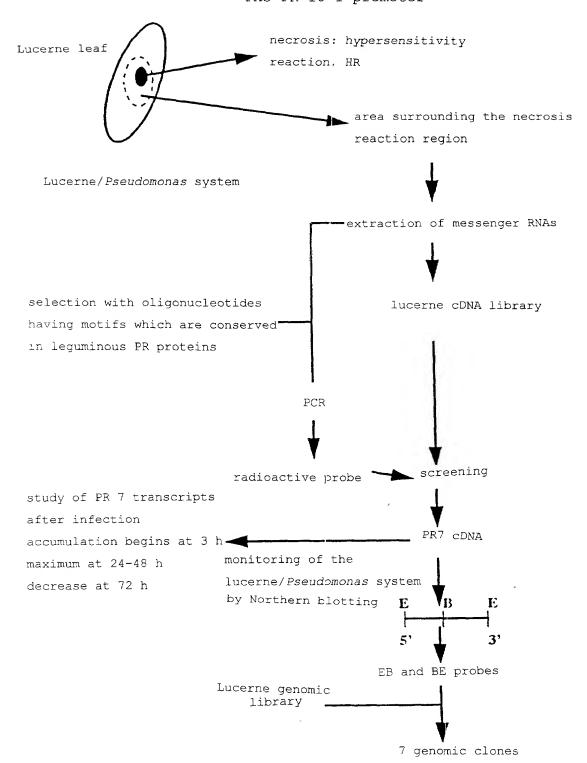
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transformed using a microbiological method including a system or vector according to one of Claims 9 to 19.

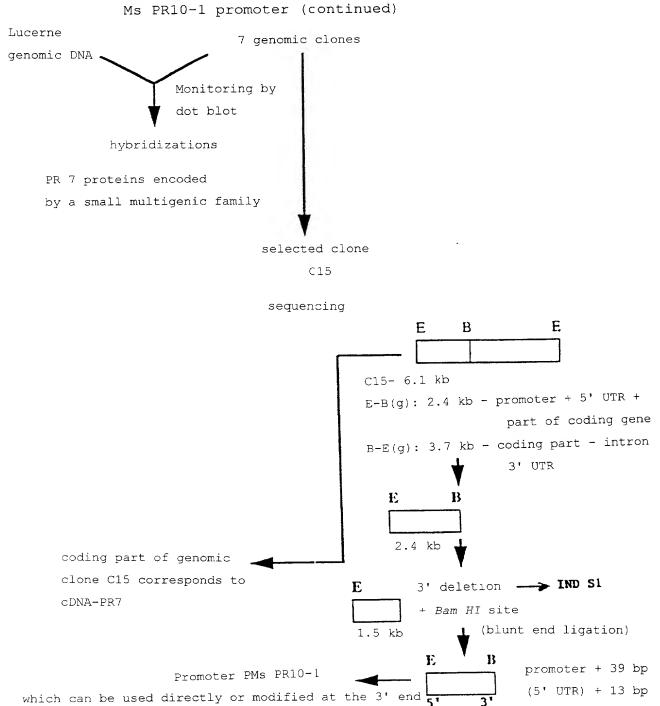
- 23. Process for obtaining plants which express a stilbene synthase gene, characterized in that cells of the said plants are transformed using a system or a vector according to one of Claims 9 to 19, the cells expressing the said gene are selected and a plant is regenerated from these cells.
- 24. Plant comprising an expression system according 10 to one of Claims 9 to 19.
  - 25. Plant comprising cells according to one of Claims 20 and 21.
  - 26. Plant which is obtained by implementing a process according to one of Claims 22 and 23.
- 15 27. Plant according to one of Claims 24 to 26, characterized in that it is a plant of agricultural interest.
  - 28. Plant according to Claim 27, characterized in that the plant is grapevine.

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FIGURE 1: Scheme for isolating the PMs PR 10-1 promoter



# FIGURE 1a: Scheme for isolating the Ms PR10-1 promoter (continued)



of the coding part (framed Fig. 5)

FIGURE 2

	SOUTHERN BLOT			
C3 9.3	E B	B	E	9.6
C12 6.5	E	B	E	6.7 (doublet ?)
C15 6.1	E	В	E	
C16 5.8	E	В	E	5.8
C13 4.8	E 	В	E	5.2
C8 4.2	E	B E		3.5
C7 2.2	E	B E		2.4
(=	lkb)			1.65 (weak signal)

FIGURE 3

gaattetteaaaaaaaagttgeeettgagaaaetaataagttaataaaetaagaeetetaa aaaaaaagttaataaactaatatgaatattCtctaaacaaaaataaaactaagaagaatat attttqcttatttaccagaaaaatactttgcttagtcaaaagaaqaaqaatattqtqaatta attatatatttaattggtgtgtctattcaagtgtttaacttaagttgaggtttattcttatg ttactaagttggagtggagaagaagactatttgcttgggaggaggaacgcccagtaqaatqt qttattattttttttttttttataaggagtagagtgtgttatgttqcttqaataatttttt ttgtaggataatgtattagacaaattagatattggaaacacgaccctgtcaaagagtacacqqt aaagggggtggtatacaaaagagtgcgtcgctctattcttcaggtcatttggtttgctacaq tttaggaaatttgggaggaaagaaataacagactgtataacgtcaaagaatgctcggttatt caggtggtagataagattaagtttcttgcttttcattgcatgggtgaaggcaaagtttgcttctct tccattcaattaccatgggtggcggcttagtccgtttaccatactggacataggctaaqaqt ttttcttttctcgtttttccattacaagttctttatgtaaatactgttttgactttqqtqtt cttcccttagtacaccttgtgctaggaaggactattttgatttggtaatatatttcatttta acctcttaaaaaaaatcaggaaaagaaaaagataaaggtcggaagtgttacctgattataa aataaatgattaaattgaaaataaagataaataactaaaatgttttctataattaagttaag aatagtagatgcaataattttgttagtccaaataataatatagttttctttaaaaataatat catccaaactcatacattaaaaatattattcaaatttatgtcacgtcacaatgagaaaaaat qqcccaacgaccttgtattacacatcatcgtcatcatcatctaaagtctaaacaatacatct tcttttcctataaatacaagactcaactccactataaatcacacaggcaaacaattaactt cttaatagtttgttatttcacacattag

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FIGURE 4

GATCCGGCTTCAATTGAGGAAATTAGAAA.CGCTCAACGTGCCAAGGGTCCGGCCACCATC CTAGCCATTGGCACAGCTACTCCGACCACTGTGTCTACCAGTCTGATTATGCTGATTAC TATTTCAGAGTCACTAAGAGCGAGCACATGACTGAGTTGAAGAAGAAGTTCAATCGCATA Tgtaagtatatattcatgcattaattcttacattcacaacatttctatacatatacga ttttagctaaattactttaggaaacttgaaaatcattttacatcagtaaccgatattcct aattaagaataaccttttataatttcttcaatgttaaatgcatgttgatcatcttcaaga atatactatatgactagtcgttggaaaactaatgtgttcatcttatttcttttacaggGT GACAAATCAATGATCAAGAAGCGTTACATTCATTTGACCGAAGAAATGCTTGAGGAGCAC CCAAACATTGGTGCTTATATGGCTCCATCTCTCAACATtACGCCAAGAGATTATCACTGC TGAGGTACCTAAACTTGGTAAAGAAGCAGCATTGAAGGCTCTTAAAGAATGGGGTCAACC AAAGTCCAAGATCACCCATtCTTGTATTTTGTACAACCTCCGGTGTAGAAATGCCCGGTG CAGATTACAAACTCGCTAATCTCTTAGGCCTTGAAACATCGGTTAGAAGGGTGATCTTGT ACCATCAAGGTTGCTATGCAGGTGGAACTGTCCTTCGAACTGCTAAGGATCTTGCAGAAA ATAACGCAGGAGCACGAGTTCTTGTGGTGTGCTCTGAGATCACTGTTGTTACATTTCGTG GGCCTTCCGAAGATGCTTTGGACTCTTTAGTTAGGTCAAGCCCTTTTTGGTGATGGGTCA GCAGCTGTGATTGTTGGATCAGATCCAGATGTCTCCATTGAACGACCCCTCTTCCAACTT GTTTCAGCAGCACAAACGTTTATTCCTAA'TTCAGCAGGTGCTATTGCGGGTAACTTACGT GAGGTGGGACTCACCTTTCACTTGTGGCCTAATGTGCCTACTTTGATTTCCGAGAACATA GAGAAATGCTTGAATCAGGCTTTTGACCCACTTGGTATTAGCGATTGGAACTCGTTATTT TGGATTGCTCACCCTGGTGGCCCTGCAATTCTTGATGCAGTTGAAGCAAAACTCAATTTA GAGAAAAAGAAACTTGAAGCAACAAGGCA'TGTGTTAAGTGAGTATGGTAACATGTCTAGT GCATGTGTCTTTGTTTATTTTGGATGAGA'TGAGAAAGAAATCCCTAAAGGGGGAAAAAGC TATCCACAGGTGACGGATTGGGGGGTACTATTCGGTTTTGGGCCAGGCTTGACCAT TGAGACCGTTGTGCTGCATAGCGTTCCTATGGTTACAAATTGAgtggaaaacggtaagag aaatgatataggggacatgtcttattgtattatcagaggaggtgctacgaaagatatqta catgtatcttcaaagttaataattagtactcctaaatcttttattcctatcctaacattg agggattgtaatttagtgattgttggagggtgcagtcacgtcaggcaagtggatgaaact gcaagtgcttgtcattctgttatcgggggatcatccatcacactggcggccgctcgagca tgcat

FIGURE 5

qaattetteaaaaaaaagttgeeettgagaaactaataagttaataaactaagaeetet aaaaaaaaqttaataaactaatatgaatattctctaaacaaaaaataaaactaaqaaga atatattttqcttatttaccagaaaaatactttqcttagtcaaaaqaaqaaqaatattqt qaattaatttqatactgatgatttttaaagctgtagatatttacgtatttaqttaaaaaa atacaattattatatttaattggtgtgtctattcaagtgtttaacttaagttgaggtt tattcttatgttactaagttggagtggagaagactatttgcttgggaggaggaacgc ccagtagaatgtgttattattttttattttttttgtaaggagtagagtgtgttatgttqct tqaataattttttttttgtaggataatgtalttagacaaataaattttgqaaacacqaccctq tcaaagagtacacggtaaagggggtggtatacaaaagagtgcgtcgctctattcttcaqq tcatttggtttgctacagtttaggaaatttgggaggaaagaataacagactgtataacq tcaaagaatgctcggttattcaggtggtagataagattaagtttcttgcttttgcatggq tgaaggcaaagtttgcttctcttccattcaattaccatgggtggcggcttagtccgttta ccatactggacataggctaagagtttttctttttctcgtttttccattacaagttctttat ataaaqqtcggaaqtgttacctgattataaaataaatgattaaattgaaaataaagataa ataactaaaatgttttctataattaagttaagagatgaaatatgtaattttcccaattat atattatgtaagtttttatttattttatatacgttgttttgctttgaaatttgagtggtc ttggaggagagaaaacaaaagagaaaagaaaaattaatagtagatgcaataattttgtt aqtccaaataataatatagttttctttaaaaaataatatcatccaaactcatacattaaaa atattattcaaatttatgtcacgtcacaatgagaaaaaatggcccaacgaccttgtatta cacatcatcqtcatcatctaaaqtctaaacaatacatcttcttttcctataaataca agactcaactccactcataaatcacacaggcaaacaattaacttcttaatagtttgttat ttcacacattaggggccagATGGAGGATCCGGCTTCAATTGAGGAAATTAGAAACGCTCA ACGTGCCAAGGGTCCGGCCACCATCCTAGCCATTGGCACAGCTACTCCCGACCACTGTGT GTTGAAGAAGAAGTTCAATCGCATATgtaagtatatatattcatgcattaattcttacat tcacaacatttctatacatatacgagtgtgctattaagtgagggtcacctccaagtgaat gaatgtttcaagcttagagaatagcttttagctaaattactttaggaaacttgaaaatca ttttacatcagtaaccgatattcctttcatttgattgtaagggcttgaagagctgttctt tqaatcatqtaqcattqctaqctataattaaqaataaccttttataatttcttcaatqtt aaatgcatgttgatcatcttcaagaatatactatatgactagtcgttggaaaactaatgt gttcatcttatttcttttacaggGTGACAAATCAATGATCAAGAAGCGTTACATTCATTT GACCGAAGAATGCTTGAGGAGCACCCAAACATTGGTGCTTATATGGCTCCATCTCTCAA CATŁACGCCAAGAGATTATCACTGCTGAGGTACCTAAACTTGGTAAAGAAGCAGCATTGA AGGCTCTTAAAGAATGGGGTCAACCAAAGTCCCAAGATCACCCATtCTTGTATTTTGTACA ACCTCCGGTGTAGAAATGCCCGGTGCAGATTACAAACTCGCTAATCTCTTAGGCCTTGAA ACATCGGTTAGAAGGGTGATCTTGTACCATCAAGGTTGCTATGCAGGTGGAACTGTCCTT CGAACTGCTAAGGATCTTGCAGAAAATAACGCAGGAGCACGAGTTCTTGTGGTGTGCTCT TCAAGCCCTTTTTGGTGATGGGTCAGCAGCTGTGATTGTTGGATCAGATCCAGATGTCTC CATTGAACGACCCCTCTTCCAACTTGTT'CCAGCAGCACAAACGTTTATTCCTAATTCAGC AGGTGCTATTGCGGGTAACTTACGTGAGGTGGGACTCACCTTTCACTTGTGGCCTAATGT GCCTACTTTGATTTCCGAGAACATAGAGAAATGCTTGAATCAGGCTTTTGACCCACTTGG TATTAGCGATTGGAACTCGTTATTTTGGATTGCTCACCCTGGTGGCCCTGCAATTCTTGA TGCAGTTGAAGCAAAACTCAATTTAGAGAAAAAGAAACTTGAAGCAACAAGGCATGTGTT AAGTGAGTATGGTAACATGTCTAGTGCATGTCTTTTGTTTATTTTGGATGAGATGAGAA TCGGTTTTGGGCCAGGCTTGACCATTGAGACCGTTGTGCTGCATAGCGTTCCTATGGTTA

FIGURE 5 (continuation 1)

## FIGURE 6

Uninduced control

Uninduced clone 2

Induced clone 2

Uninduced clone 3

Induced clone 3



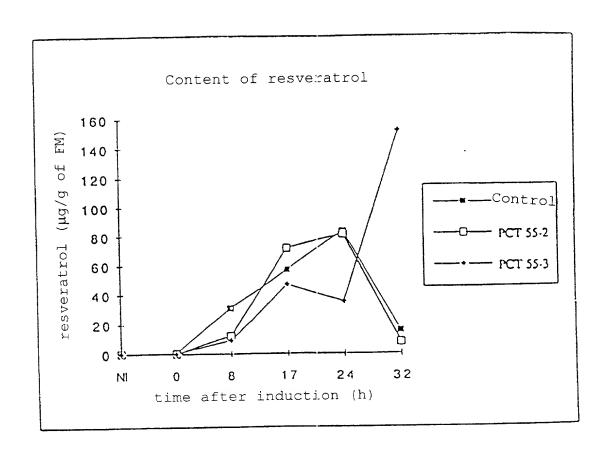
FIGURE 7

	CONTROL					CLONE 2					CLONE 3												
_	uninduced	immediately after	induction	8 h after induction	17 h after induction	24 h after induction	32 h after induction	-	nnınduced	immediately after	induction	8 h after induction	17 h after induction	24 h after induction	32 h after induction		uninduced	immediately after	induction	8 h after induction	17 h after induction	24 h after induction	32 h after induction
						(j.) 1			V in								·	\ \frac{1}{2}				i.	- orthographic

PCT/FR99/00316

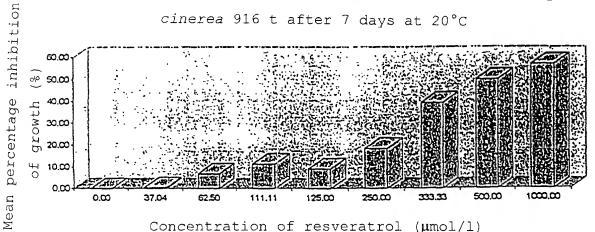
10/14

FIGURE 8



#### FIGURE 9

Inhibition of the growth of the mycelium of Botrytis cinerea 916 t after 7 days at 20°C



Concentration of resveratrol  $(\mu mol/1)$ 

FIGURE 10: Photographic plate 1

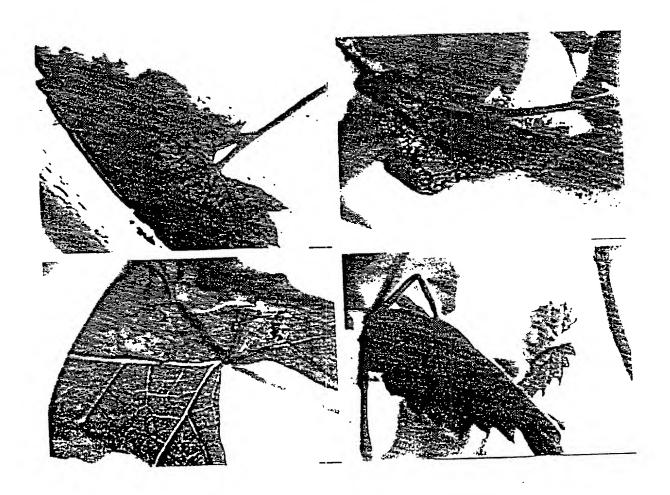


FIGURE 11: Photographic plate 2

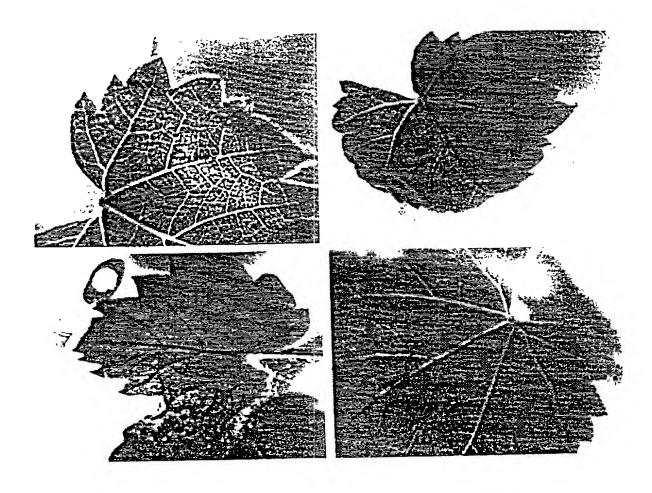
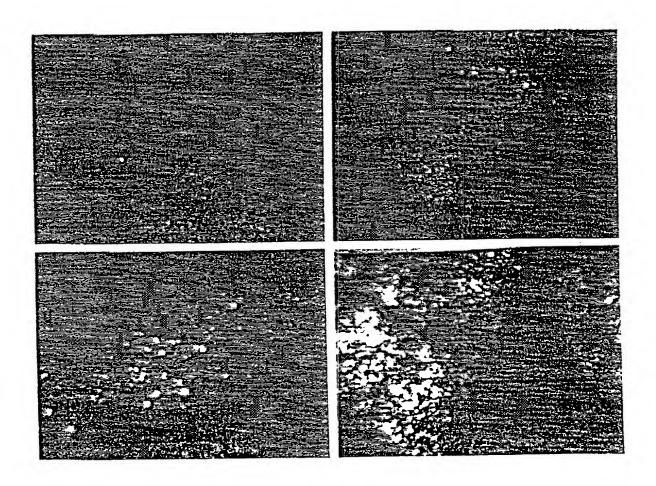


FIGURE 12: Photographic plate 3



As a below-named inventor, I hereby declare that:

DECLARATION FOR PATENT APPLICATION

s a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and sole inventor (if plural names are listed below) of the NUCLEIC ACID COMPRISING THE SEQUENCE subject matter which is claimed and for which a patent is sought on the invention entitled

OF A STRESS-INDUCIBLE PROMOTER AND A SEQUENCE OF A GENE ENCODING A STILBENE SYNTHASE

the specification of which: (check one)							
PCT/FR99/00316 , and was amended	d on19(ıf applıca August 14, 2000 a	s U.S. Serial No. 09/622,	257				
I acknowledge the duty to disclose information which is mater Prior Foreign Application(s) I hereby claim foreign priori inventor's certificate listed below, or § 365(a) of any PCT internal listed below and have also identified below any foreign applicate priority is claimed	ty benefits under 35 USC § 11 ational application which designation	19(a)-(d) or §365(b) of any foreign application ated at least one country other than the United S	States of America,				
98/01742	<u>F'RANCE</u>	13/February/1998	I [ ]				
(Application No )	(Country)	(Day/Month/Year Filed)	Yes No				
I hereby claim the benefit under Title 35, United States Code §	119(e) of any United States prov	visional application(s) listed below:					
Application No		Filing Date					
I hereby claim the benefit under 35 U S C § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the mannel provided by 35 U.S.C. § 112, first paragraph, I acknowledge the duty to disclose material information as defined in 37 CFR § 1 56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application.							
(U S Application Serial No )	(US Filing Date)	- (Statuspatented, pending, aba	ndoned)				
I hereby appoint George Vande Sande, Registration No. 17,2 Townsend M Belser, Jr., Registration No. 22,956, Morris Liss, I	Registration No 24,510; Martin A	Abramson, Registration No 25,787; George R I	Pettit, Registration				

No. 27,369, Elzbieta Chlopecka, Registration No. 32,767, Eric J. Franklin, Registration No. 37,134; Jeffri A. Kaminski, Registration Number 42,709, and William E. Curry, Registration No. 43,572, my attorneys with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon

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### DECLARATION FOR PATENT APPLICATION

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